THIS NUMBER COMPLETES VOLUME 14

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EXPERIMENTAL CELL RESEARCH

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

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VOLUME 14 · JUNE 1958 · NUMBER 3

ACADEMIC PRESS INC., PUBLISHERS
NEW YORK and LONDON

EXPERIMENTAL CELL RESEARCH

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AUTORADIOGRAPHY OF NUCLEOLAR CONTENTS

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Received August 21, 1957

KNOWLEDGE of the internal morphology of nucleoli (i.e. [1, 5]) has progressed faster than that of their internal physiology, due to the better resolution of microscopical as compared with chemical techniques. The latter include autoradiography which, though having contributed considerably to the study of nucleolar function, has not been particularly used for intranucleolar work. Some observations of such character, gathered with Kodak AR 10 film under conditions for high resolution, are reported here. Because of the dimensions of the structure studied some of these observations may require confirmation by other techniques.

Two types of nucleolar autoradiographs (ARGs) have been seen. In the first, photographic grains are peripheral to the nucleolus. These are produced by the chromatin associated with the nucleolus, and also by the periphery of the nucleolus itself (there is no membrane between them). Geometrically it is obvious that to produce peripheral ARGs either or both of these two sources must be sectioned as annuli. This type of ARG was first observed with phosphorus-32 in the mouse oocyte [10]. It was later encountered in the salivary gland nuclei of *Drosophila* with RNA-^{32}P [15], with phenylalanine-^{14}C in proteins, and with adenine-^{14}C in RNA purines [13]; and with protein sulphur-35 in the chick embryo [14]. It has now been found in the explanted amphibian somite with the last two tracers. It also occurs in mouse embryos, from oocytes to blastocysts, using precursors of proteins and/or nucleic acids [4]. Figs. 1 and 2 show these ARGs in amphibian embryos, with proteins or nucleic acids labelled *in vivo* with arginine- or adenine-^{14}C, respectively. Note the similarity of Fig. 2 with Fig. 3 in [14]. Labelling was extended over a day. The arginine is in part in residual proteins, as it resists the extraction of histones with acids. Some of the ^{14}C from adenine is actually in DNA; it was removed by total extraction of nucleic acids but not by cold trichloroacetic (5 minutes) or Feulgen hydrolysis.

The above autoradiographical evidence shows conclusively the involvement of the nucleolar periphery in the metabolism of nucleic acids and pro-

[1] Research worker of the British Empire Cancer Campaign.

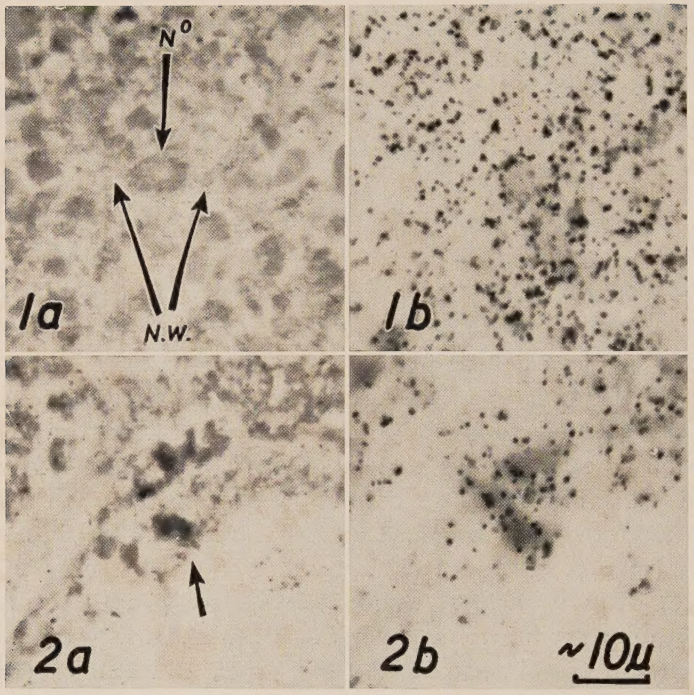


Fig. 1.—Somite cell of *Xenopus* with proteins labelled with L-arginine-^{14}C. Methyl green–pyronin. (*a*) Nucleolus (n^o) with DNA shell; *n.w.*, nuclear wall. Phase contrast. (*b*) Autoradiograph on the shell.

Fig. 2.—Chordal sheath cell of *Xenopus* with nucleic acids labelled with adenine-8-^{14}C. Methyl green–pyronin. (*a*) Nucleolus with chromatin condensed around. (*b*) Autoradiograph on the periphery of the nucleolus.

Figs. 3–7.—Somite cells of *Xenopus* with proteins labelled with L-arginine-^{14}C (Figs. 3 to 6), or with nucleic acids labelled with adenine-8-^{14}C (Fig. 7). ARGs on the periphery of nucleoli partly correspond to associated chromatin. Figures (*a*) are photographs at tissue level; figures (*b*), at one level in the emulsion; and figures (*c*) are the same as figures (*a*), with most grains in the emulsion drawn in. Methyl green–pyronin. Some photos taken under phase contrast.

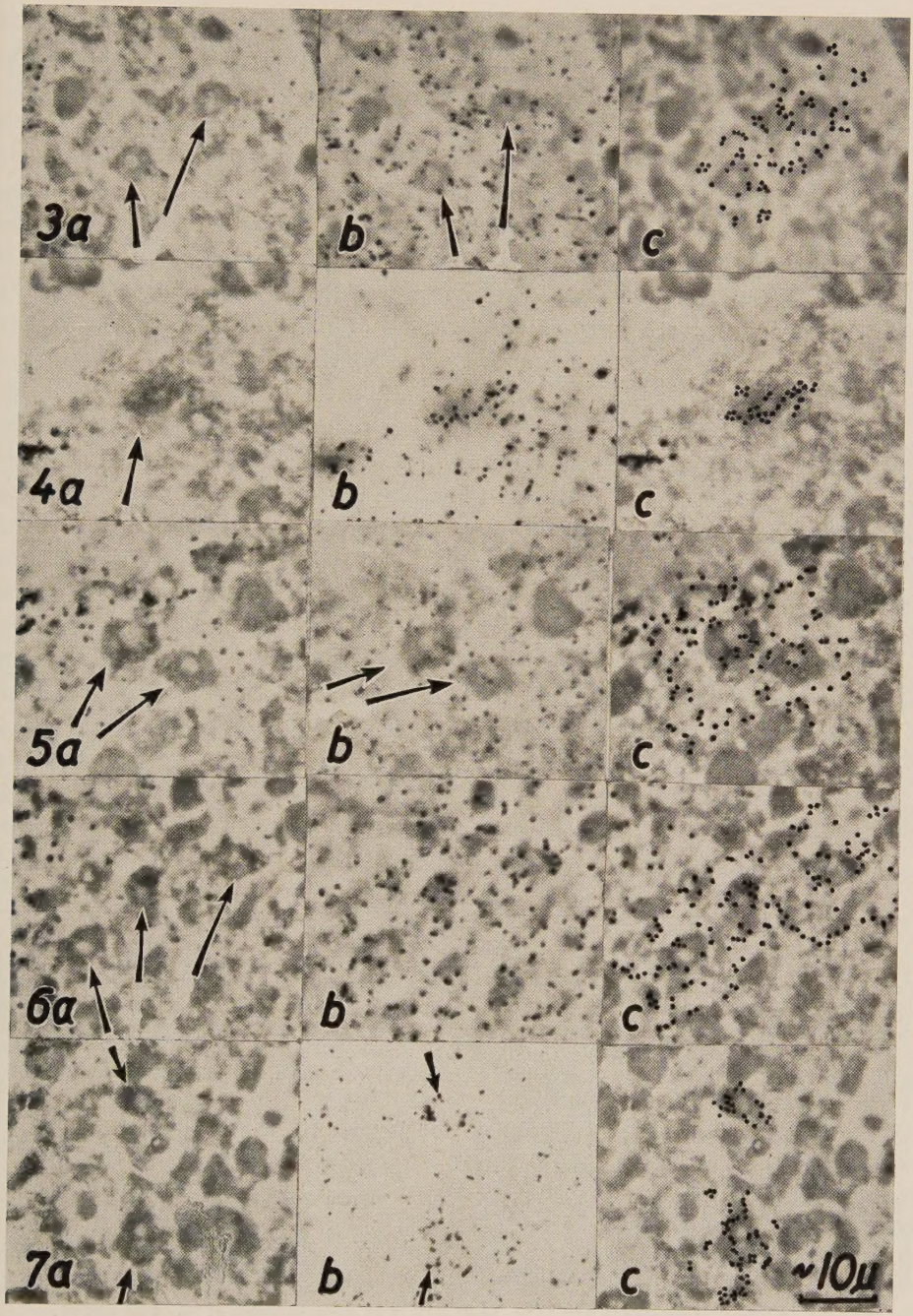
Fig. 3.—Two vacuolated nucleoli. ARGs on the nucleoli correspond to the body except the vacuoles. ARG's also on the nuclear walls.

Fig. 4.—One nucleolus with two vacuoles, and its ARGs as in Fig. 3. Below the nucleolus is condensed chromatin also giving an ARG.

Fig. 5.—Two vacuolated nucleoli and their ARGs. Note ARGs on nuclear wall, and not in the nuclear space.

Fig. 6.—Three vacuolated nucleoli. ARGs as in Fig. 5.

Fig. 7.—Two nucleoli, one (above) with one vacuole, and one (below) with two vacuoles, and their ARGs as in Fig. 3.


3a
b
c
4a
b
c
5a
b
c
6a
b
c
7a
b
c
~10μ

teins. Related evidence is that of RNA forming under the DNA shell of pronuclear nucleoli [3], and of the richness of phosphatase in both these layers (cf. [9]).

Secondly, there are ARGs given by the body of the nucleolus (references as before; [6]). In general the "reality" of these ARGs is established beyond doubt, but in any particular instance some difficulty may be met in deciding if the ARG originates in the nucleolus or in the apposed chromatin. For studying this point, advantage has been taken of the sizeable vacuoles in nucleoli of *Xenopus* somites. ARGs corresponding to the whole nucleolus except the vacuoles are frequently observed (Figs. 3 to 7): it is difficult to imagine these ARGs being produced by anything but the body of the nucleolus.

Internal autoradiographical detail can be further elucidated in squashes of salivary gland nucleoli of *Drosophila*. Larval proteins were labelled with methionine-^{35}S from the onset of feeding; in this way the tracer measures concentrations, and not rates of incorporation as with shorter periods of labelling. After Feulgen hydrolysis, which leaves behind mainly non-histone (residual) proteins, ARGs can be seen over all the nucleolus except for its vacuoles or DNA inclusions (Figs. 8 to 14). Vacuoles may be often missed in these preparations but the absence of definite ARGs associated with the observed nucleolar DNA is constant (out of 52 nucleoli with DNA scored).

There is some indication of an ARG around the vacuoles observed in Figs. 8*a* and 11*a*. This provides some basis for assuming that in the other figures, where vacuoles are not observable under phase constrast, the annular ARGs found on about the centre of all these nucleoli point in fact to unseen vacuoles. If so, in all the nucleoli illustrated the vacuole and the DNA

Figs. 8–14.—Nucleoli of salivary glands of *Drosophila* with residual proteins labelled with methionine-^{35}S. Only the bulkier DNA inclusions, stained with basic fuchsin, can be seen under the emulsion and are indicated with arrows. In nucleoli of Figs. 8*a* and 11*a* vacuoles are observable and it is likely that they occur in the others (see text). Figures (*a*) are phase contrast photographs at tissue level; figures (*b*), bright field photographs at emulsion level. Grain size varies between photographs due to variations in the copy of negatives. The scale of magnification for Fig. 10 and 11 is inserted in Fig. 10*b*; for the other figures, in Fig. 14*b*.

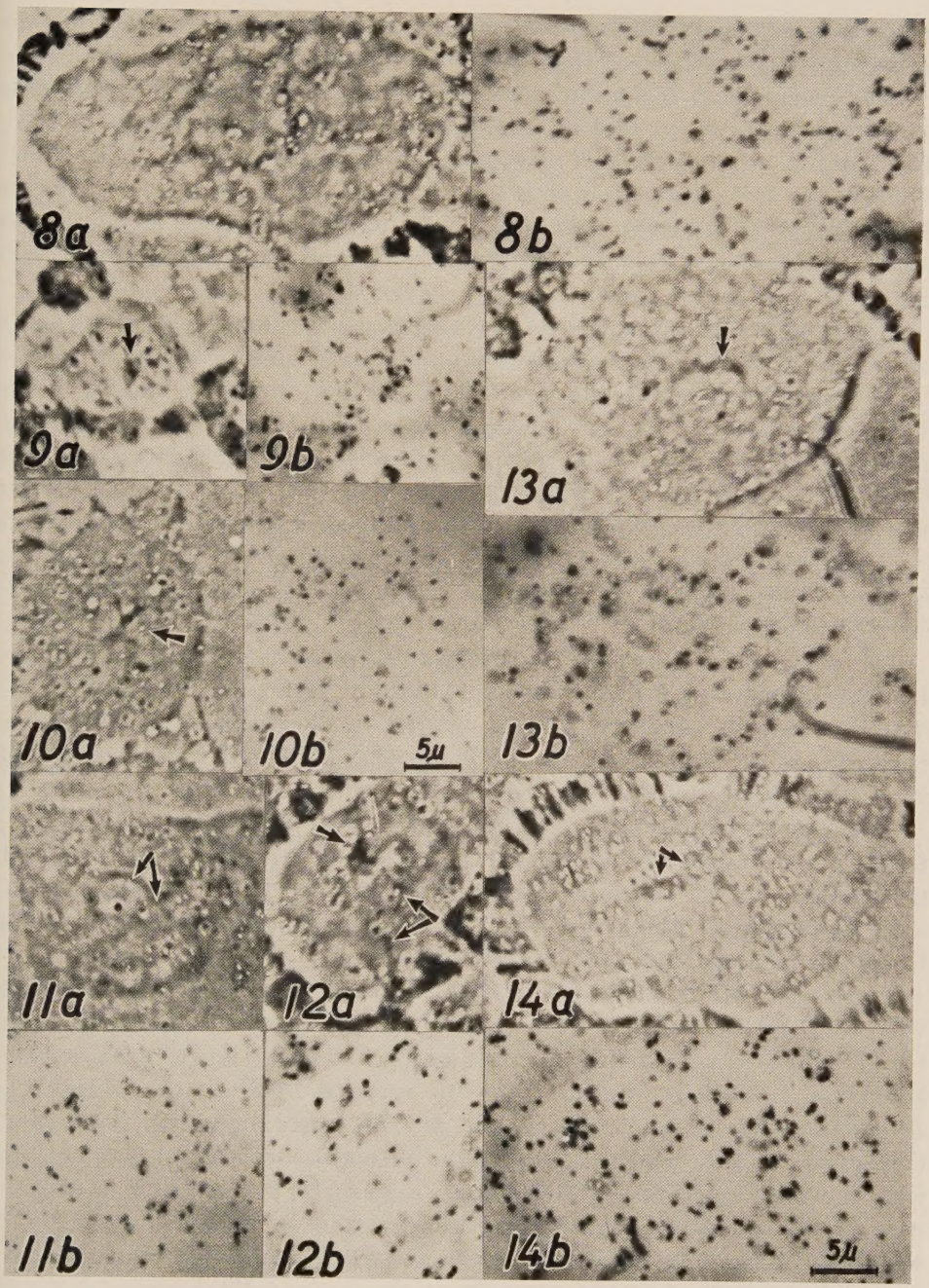
Fig. 8.—No DNA observed. There is a vacuole with peripheral ARG.

Figs. 9, 10, 13.—DNA bodies showing no ARG.

Fig. 11.—Elongated DNA body, with no ARG, and a vacuole with peripheral ARG. The body is seen as cutting the vacuole in two halves.

Fig. 12.—DNA body, with no ARG, linked by a DNA thread to the basal part of chromosome 3L. Two focused segments of the thread pointed by double arrows. Chromocenter at bottom left.

Fig. 14.—DNA body, with weak ARG (?). Note ARGs on chromosome at left and above.


8a
8b
9a
9b
13a
10a
10b
5μ
13b
11a
12a
14a
11b
12b
14b
5μ

inclusion would be superimposed; this is the result of chance, as it is easy to find inactive inclusions lying at the side of vacuoles.

The concentration of sulphur in the residual proteins of the whole nucleolus is of the same order as that in whole chromocenters or chromosomes [12]. The types of residual proteins in all these constituents need not be the same. Nonetheless, the comparison with the chromocenter is of interest because this includes the nucleolar organizer. The similarity in the concentration of protein sulphur in nucleoli and chromosomes emphasizes the differences in metabolic rates which may be found between these two components.

In *Drosophila* the salivary gland nucleolus has been described as frequently containing vacuoles, usually central, around which DNA bodies are disposed [9]. Feulgen positive threads unite the nucleolus and these bodies to the chromocenter [7, 9]. We have been able to confirm these observations in sections; further we have noticed in squashes that the threads can join the chromosome arms as well as the chromocenter (Fig 12*a*). It is very clear in squashes that there is no DNA shell round these nucleoli (cf. [7]), as there is in other interphase nuclei. The chromatin associated with the nucleolus is here in the form of the persistent chromosomes and heterochromatic chromocenters.

The vacuoles of *Drosophila* seem empty of proteins or nucleic acids [9]. In electron micrographs Lowman finds that the vacuoles are not empty [8]; that they contain materials similar to that in chromosome bands, as he contends, is not proved by the evidence presented and is improbable in view of all other evidence.

DNA seems of rather general occurrence inside nucleoli (references in [9]; for the chick embryo see [2, 14]). It is possible then that some DNA surrounds the vacuoles in the amphibian nucleoli of Figs. 3 to 7, in which case the autoradiographical picture in terms of proteins should correspond to that found in the larger nucleoli of *Drosophila*.

From these observations the relationship of DNA with proteins seems to vary with the different sites. This appears most simply if one considers the results obtained with methionine-^{35}S, which has been shown to be detected by ARGs as protein sulphur [11]. The internal DNA of the nucleolus of *Drosophila* appears to be not at all, or little, associated with non-histone (residual) proteins, while the DNA in the chromosomes around the nucleolus is as a whole associated with residual proteins [12]. The latter is also the case in the amphibian somite (see above), and in the chick embryo [14] where the DNA in the shell around the nucleoli is associated with total proteins, a good deal of which are residual. Similarly, DNA is associated with

RNA in the chromosomes (as shown by stain and ARGs) and in the nucleolus-associated chromatin (by ARGs) (unpublished), but not in the intranucleolar bodies ([9], by stain).

SUMMARY

Some observations on the proteins and nucleic acids of nucleoli, gathered with high resolution autoradiography, are presented.

I wish to thank Professor C. H. Waddington for discussion of the manuscript and Mr. D. W. Pinkney for assisting with the photography.

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VARIATION IN RESPIRATION OF EARLE'S STRAIN L CELLS

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Received August 21, 1957

Earle's strain L cells [3] were derived from subcutaneous connective tissue of a 100-day-old C3H mouse and grown by the tissue culture technique. After 291 days in culture the cells were treated for 111 days with 1.0 gamma methylcholanthrene per ml culture medium. In 1948, Sanford [11] isolated one cell from a culture of strain L and succeeded in establishing a pure clone from the single cell.

One might expect that subsequent progeny of this single cell would be relatively uniform in appearance and have a stable metabolism. However, respiration of Earle's strain L cells on various occasions has been found to be different. As indicated in previous reports [5, 8, 6] respiration was found to be 16.2, 4.8 and 9.4 mm^3 O_2 mg/hour, respectively.

A year before the present report, a culture of strain L was obtained from Dr. D. M. Pace which had numerous irregular shaped, multi-nucleated giant cells (Fig. 1). Since the culture presented such an unusual morphology, it was decided to grow it separately from other cultures of strain L in order to find out if its characteristics were permanent. Most strain L cultures examined in other laboratories were found to contain a few giant cells; however, subcultures of the original irregular culture contained a much greater number even after continuous cultivation for more than a year. Cultures of strain L which had an increased number of giant cells were arbitrarily called strain L-X to distinguish them from cultures of strain L cells which presented a relatively uniform appearance.

In the course of investigation, the effect of pH on anerobic glycolysis, the adjustment of pH by the cells and the survival of cells for short periods of anaerobiosis were studied. This was done so that better experimental conditions could be attained. The primary objective of this study was to compare respiration of strain L-X to that of strain L. It was thought that there might be metabolically different cells in cultures of strain L and this might explain why we had obtained different values for respiration in past experiments.

MATERIAL AND METHODS

All cultures had been grown in our laboratory at least three months in T-60 flasks in a medium of 40 per cent Earle solution, 40 per cent horse serum and 20 per cent chick embryo extract (50:50) which was filtered through a Selas filter. About a year before metabolic studies were started for the present experiments, both strain L and strain L-X were transferred to and grown in a hydrolysate medium [7] which was supplemented with 10 per cent pooled serum. Pooled serum contained equal parts of human, horse, sheep, and cow serum. Medium was changed three times weekly and cultures had to be subcultured about every week and a half. Culture medium was renewed the day before determinations were made.

A cell suspension for metabolic determinations was prepared in a phosphate buffered balanced saline solution with 10 per cent serum as has been previously described [7]. The pH of the cell suspension was checked on a Beckman pH meter and if it varied outside the limits of pH 7.2–7.4 it was adjusted to about pH 7.3 with either 1.75 per cent Na_2HPO_4 or 2.28 per cent NaH_2PO_4. The number of viable cells [9] in a suspension was obtained by adding 0.1 ml of 20 mg per cent Erythrosin B to a 1.0 ml aliquot of the suspension and then cells were counted on a hemacytometer. Both pH and per cent viable cells were determined at the beginning of a metabolic experiment and again at the end of that experiment. Values for pH or cell count were taken as the mean of the initial and final determination. After shaking cells on a Barcroft–Warburg Respirometer for an hour they tend to clump and thus introduce error in the final hemacytometer count. For this reason, the cells were redispersed at the end of each experiment by passing them back and forth through a pipette.

Respiration (Q_{O_2}) was determined by the Direct Method of Warburg [13] at 37.5°C in air. Small reaction vessels were used (7.5 ml) which contained about 2×10^6 cells each. Flasks and manometers were rocked at 100 rpm for 15 minutes to equilibrate for temperature and then for exactly one hour to make a determination.

The stimulatory effect of sodium succinate on respiration [10] was also checked. Respiration was determined for one-half hour; sodium succinate solution was dumped from the side arm of the reaction vessel in an amount to yield a final concentration of 0.2 *M* and then respiration was noted for another half hour. The effect of succinate on respiration for the second half hour was compared to respiration during the first half hour. Thus, each reaction vessel served as its own control.

Lactic acid was determined chemically by the Barker–Summerson Method [1]. Photometric standard graphs were made with lithium lactate. Lactic acid, the concentration of which was determined by titrating with standardized sodium hydroxide, was also used. No essential difference was found between the two lactate standards.

Aerobic glycolysis (Q_{LA}^{Air}) was measured under conditions like those used for respiration except that no potassium hydroxide was used in the center well of the reaction vessel. Determinations were run in pairs so that one lactate measurement was made immediately after equilibration for temperature and another, one hour later. The difference between the two values was taken as glycolysis. Anaerobic glycolysis ($Q_{LA}^{N_2}$) was measured like aerobic except that the cell suspension and reaction vessels were gassed with 99.6 per cent nitrogen.

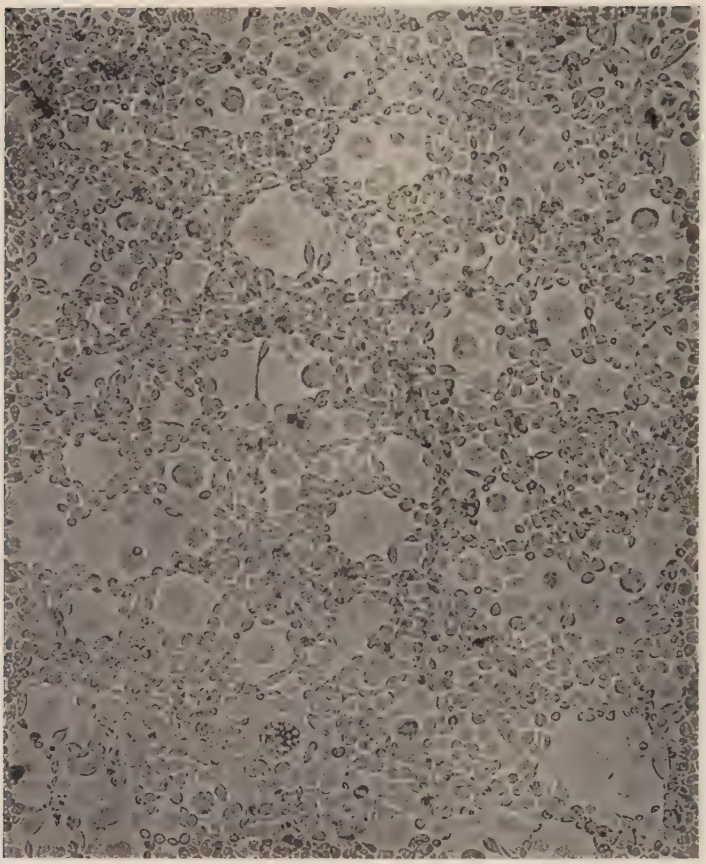

Fig. 1.—Cultures of strain L showing giant, multinucleated cells. — *A*. An old culture of strain L showing numerous giant cells (courtesy Dr. D. M. Pace). — *B*. A new culture of strain L at higher magnification showing one giant cell.

All metabolic quotients were based on live rather than total cell count because it had been shown that dead strain L cells under similar experimental conditions did not respire or glycolyze [9]. In order to conform to methods of other workers, viable cell count was converted to dry weight when 10^6 cells weigh 0.46 mg [6].

RESULTS

Warburg [13] found that lactic acid formation of tissue slices increased with increase in pH. This phenomenon was investigated for strain L in hopes that optimal pH for glycolysis could be determined. As pH was raised, more lactic acid was formed (Fig. 2). Within the range of pH 6.9–7.9 strain

Fig. 1 *B*.

L-X will survive and grow. However, three times as much lactic acid was formed at pH 7.9 than at pH 6.9. Replicate experiments were made on strain L and strain L-X with similar results.

An unexpected result was observed when pH was determined at the beginning and end of an experiment under anaerobic conditions. The cells tended to adjust the medium to pH 7.2. If pH was above 7.2 they lowered it by increased lactic acid formation (Fig. 2). If pH was below 7.2 they raised it, apparently, by exchange of intracellular potassium for extracellular hydrogen. Evidence for this mechanism was obtained by making the cells work against a gradient. When extracellular potassium chloride was increased from 0.3 to 0.6 g per liter the cells were not able to raise pH. These observations indicate that the cells alter their metabolism and exchange cations in order to adjust pH of

their environment. In short, this phenomenon appears to be a homeostatic mechanism for regulation of pH.

All metabolic quotients used to compare strain L with strain L-X were obtained within a range pH 6.9–7.5. Any value obtained that lay outside this range was discarded since lactate formation varied considerably with change of pH.

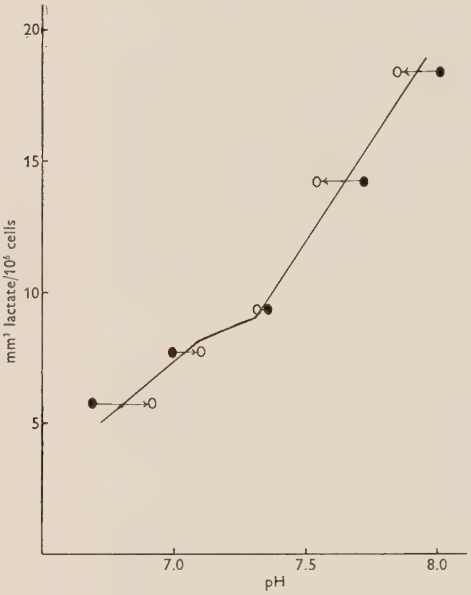

Fig. 2.—Effect of pH on anaerobic lactate formation of strain L in phosphate buffered balanced saline. Each point is a mean of two determinations. Shaded circle was initial pH; open circle was pH an hour later.

The two subcultures differed metabolically (Table I). Results were interpreted statistically by the *t* test for experiments involving independent groups [4]. Respiration of strain L ($Q_{O_2} = 13.6$) was found to be higher than that for strain L-X ($Q_{O_2} = 4.7$). The difference was very significant ($P < 0.001$). Aerobic glycolysis was also found to be very significantly different ($P < 0.001$). It was low for strain L ($Q_{LA}^{Air} = 5.8$) and moderate for strain L-X ($Q_{LA}^{Air} = 13.3$). There was no apparent difference in anaerobic glycolysis which was found to be moderate for both subcultures. Sodium succinate stimulated respiration of both subcultures. The per cent increase of respiration over control values was greater for strain L-X (210 per cent) than for strain L (89 per cent).

Exposing the cells to an atmosphere of nitrogen for an hour did not kill a significant number of them (Table II). Actually, fewer cells were killed under anaerobic conditions (0.5 and 4.0 per cent) than under conditions used

TABLE I. *Comparison of metabolism of strain L and strain L-X.*

	Strain L			Strain L-X			
	No.	pH	Q	No.	pH	Q	P
Respiration	19	7.49	13.6	13	7.39	4.7	<.001
Succinate effect	14	7.36	89 %	15	7.31	210 %	—
Aerobic glycolysis	20	7.33	5.8	11	7.39	13.3	<.001
Anaerobic glycolysis	18	7.32	9.1	7	7.26	11.7	>.20
Fermentation excess			−18.1			+18.7	
Pasteur effect			+ 3.3			− 1.6	

All Q values expressed as mm^3/mg dry wt/hr.
Succinate effect, % increase in respiration when 0.2 *M* sodium succinate added; fermentation excess, $(Q_{LA}^{N_2} - 2\,Q_{O_2})$; Pasteur effect, $(Q_{LA}^{N_2} - Q_{LA}^{Air})$.

TABLE II. *Loss of viable cells under aerobic and anaerobic conditions of experimentation.*

	No. expts.	Initial % viable	Final % viable	% Loss
		Strain L		
Q_{O_2}	4	73.8	66.8	−9.0
Q_{LA}^{Air}	4	71.0	68.0	−4.0
$Q_{LA}^{N_2}$	3	75.5	74.6	−0.5
		Strain L-X		
Q_{O_2}	2	76.0	72.6	−5.0
Q_{LA}^{Air}	2	79.9	80.3	+0.4
$Q_{LA}^{N_2}$	2	79.9	76.2	−4.0

to determine respiration (9.0 and 5.0 per cent). Since the cells were shaken on the Barcroft-Warburg respirometer for an hour and then redispersed through a pipette, it must be concluded that these manipulations did not have an unduly adverse effect on them.

DISCUSSION

As previously stated, the primary objective of this study was to compare respiration of strain L-X to that of strain L. The difference in metabolic quotients of the two subcultures could not be accounted for by tissue culture

conditions or by experimental procedure. However, low respiration and moderate aerobic glycolysis does appear to be associated with the giant multinucleated cells found in some cultures of strain L. Respiration can vary with the number of these cells found in a culture.

Strain L cells give rise to tumors when injected into C3H mice [12] but their overall metabolism does not conform [6] to the metabolic criteria of malignancy which has been advocated by Burk [2]. In some respects strain L-X met Burk's criteria in that respiration was low, aerobic glycolysis was moderate and there was a definite fermentation excess ($Q_{LA}^{N_2}$—Q_{O_2}). However, they failed to conform to the criteria in that there was no Pasteur effect ($Q_{LA}^{N_2}-Q_{LA}^{Air}$). In addition there apparently was an adequate functional cytochrome reserve as indicated by the stimulatory effect sodium succinate had on respiration.

Our first hypothesis on recognizing the giant multinucleated cells was that they were caused by virus infection. It was thought that a virus may have been introduced with chick embryo extract. A series of young chicks were injected with several cultures of strain L-X but no symptoms appeared within two weeks and nothing pathological was found on autopsy. Following these experiments an attempt was made to prepare an antisera in rabbits. Cells from cultures of strain L-X were removed by centrifugation and then the medium was injected into rabbits. Five ml of culture medium was injected intravenously or intraperitoneally every other day for 10 days. After two weeks the rabbits were bled by heart puncture and the serum was used instead of pooled serum as part of the tissue culture medium. Some strain L-X cultures were maintained on the rabbit serum medium for two weeks but the giant multinucleated cells continued to appear. These observations, though not considered as definite proof, do tend to indicate that the giant cells were not the result of virus infection.

The results reported here were obtained under experimental conditions slightly different from those used in previous metabolic studies on strain L [5, 6, 8]. For example, the cells for the present study were grown in an hydrolysate medium with pooled serum, whereas, 40 per cent Earle solution: 40 per cent horse serum : 20 per cent chick extract was used previously. The metabolism of other cell strains, such as HeLa, can be altered by changing the type of serum used for culturing [7]. From this report one might expect that the metabolism of strain L would vary with the type culture medium used. Another way in which this report differs from past experiments is that all values were expressed in terms of live cells. As a consequence most of the quotients reported here are higher. In past experiments Krebs-

Ringer-Phosphate solution or neutralized horse serum [14] was used to suspend the cells for a run on the Barcroft-Warburg Respirometer. A solution whose calcium, potassium and magnesium content closely simulated interstitial fluid [15] formed 90 per cent of the suspending solution used in the present study.

SUMMARY

An unusual culture of strain L cells was found and was subcultured for more than a year in an hydrolysate medium containing 10 per cent serum. These cultures were unusual in that they contained many giant multinucleated cells as contrasted to only a few giant cells found in regular cultures of strain L cells. Metabolic determinations were made on subcultures of both regular and irregular strain L. The irregular subcultures were different metabolically in that respiration was lower, aerobic glycolysis was higher and per cent increase in respiration was greater when sodium succinate was added.

All cultures of strain L tended to raise or lower pH of medium to 7.2. The mechanism for lowering pH was by increased lactic acid formation. All determinations were based on live cell count using erythrosin B. Subjecting the cells to anaerobic conditions did not kill a significant number of them.

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A CYCLIC STAINING BEHAVIOUR OF THE CHROMOSOMES DURING MITOSIS AND MEIOSIS

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Received August 22, 1957

When sections of root tips and anthers which have been fixed in Lewitsky's fluid are stained in a mixture of orange G and aniline blue (Mallory's stain [10]), it has been found that the chromosomes at interphase stain differently from those undergoing either mitosis or meiosis. This cannot be attributed to changes either in the nucleic acids, or in the gross physical nature of the chromosomes. It is inhibited by certain fixatives.

The present paper deals with the staining reactions of the chromosomes, cytoplasm and nucleoli in the meristematic cells of roots, in pollen mother cells and in pollen grains during mitosis and pollen development. Similar staining has been observed in the meristematic cells of the seven species studied.

Histochemical and biochemical evidence to be presented in a subsequent paper [9] indicate that the behaviour is associated with lipid material on the chromosomes. The effects of fixation and of various treatments on the staining reaction will also be discussed in this later communication.

MATERIALS AND METHODS

Root tips from the following plants were investigated: *Fritillaria falcata*, *Scilla sibirica*, *S. campanulata*, *Trillium grandiflorum*, *Paris polyphylla* and *Vicia faba*. Anthers of *Tradescantia bracteata* and *T. paludosa* were used for the study of meiosis, pollen grain mitosis and pollen development.

The tissue was fixed in Lewitsky's fluid consisting of 1 per cent chromic acid and 10 per cent formalin in equal volumes, was embedded in paraffin wax and sectioned at 6 μ.

A simplified form of Mallory's stain [12] was prepared by dissolving 2 g of orange G and 0.5 g aniline blue (water sol.) in 100 ml of citrate buffer (Hopkins and Williams) at pH 3. The wax was removed from the sections which were then taken through graded dilutions of alcohol to water and stained for 2–3 minutes, after which they were rinsed for at least 30 seconds in the citrate buffer at pH 3. The preparations

Fig. 1.—Section showing a developing lateral root in a primary root of *Vicia faba*; stained in orange G–aniline blue after fixation in Lewitsky's fluid. The interphase nuclei in the lateral root stain blue except those in a peripheral zone which stain yellow.

were blotted carefully, rinsed rapidly in equal volumes of absolute ethanol and tertiary butanol and then left in tertiary butanol for 2–3 minutes prior to mounting in Euparal.

Staining Behaviour in the Meristematic Cells

The colouration of the chromosomes by orange G–aniline blue changes during the mitotic cycle. They stain yellow from mid-prophase to the end of anaphase but blue in other phases, while the heterochromatic regions are stained yellow at all stages of telophase, interphase and mitosis. The nucleoli stain yellow but the change in colouration shown by the chromosomes as they pass through prophase does not appear to be related to the dissolution of the nucleoli since the chromosomes stain yellow before this occurs. The cytoplasm stains greyish, presumably by reaction with both dyes, throughout the mitotic cycle. In contrast the spindle stains blue whereas the nuclear sap remains colourless.

A marked deviation from this cyclic staining behaviour occurs in two regions of the root: one a narrow layer of peripheral cells and the other a zone of cells adjacent to the root cap. In these the interphase nuclei, as well as the chromosomes in mitosis, invariably stain yellow under our conditions of fixation. Likewise the cytoplasm in these regions shows a greater affinity for the yellow dye.

At first sight this zonal staining might appear to be due to diffusion, as a consequence of fixation, of the substance that binds the orange G. This seems unlikely in view of some observations which were made on developing lateral roots in *Vicia faba*. Sections were cut of primary roots in which were found young lateral roots still embedded in the tissues of the parent root. Although the peripheral layer of these lateral roots was not the outermost tissue in the specimen it showed the zonal staining behaviour seen in this region in adult roots (see Fig. 1).

Staining Behaviour at Meiosis

Cyclic changes in staining also occur in the chromosomes during meiosis. The chromosomes stain blue at leptotene and diplotene but acquire an affinity for the yellow dye during diakinesis. The yellow-stained nucleoli have almost completed their dissolution by this time, so that meiosis differs from mitosis in this respect.

The cytoplasm stains grey, the spindle blue and the nuclear sap is unstained as in meristematic cells of the root. At the second division the chromosomes stain as in mitosis and in the tetrads the four interphase nuclei stain

blue. No heterochromatic regions were detected either at leptotene or in the interphase nuclei in the tetrads; this is scarcely surprising since these regions are extremely small in the species of *Tradescantia* examined.

Most nuclei in the anther wall stain blue, but sometimes a few stain yellow which is perhaps due to uneven penetration of the fixative.

Staining Behaviour during Mitosis and Development of Pollen Grains

The developing pollen grains of two diploid species of *Tradescantia* were examined. The sequence of changes which lead to differentiation of the generative and vegetative nuclei in the pollen of this plant are well known which in many respects are typical of most Angiosperms (cf. [8]).

The staining behaviour of the primary nucleus, the nucleoli and chromosomes during mitosis is as described for the meristematic cells in roots. Thus the primary nucleus stains blue at interphase, the nucleoli are coloured yellow; the chromosomes stain yellow in mid-prophase and retain this property till completion of anaphase.

Of the two daughter nuclei resulting from mitosis, the vegetative nucleus lies innermost in the cell. Soon it expands and enters a resting state; it stains blue for most of the remaining part of pollen grain development. Slightly before anthesis it ceases to stain at all.

Following the expansion of the vegetative nucleus the generative nucleus likewise stains blue. Apparently its interphase is transitory since an intermediate colouration between blue and yellow is most commonly seen at this time. This supposition is supported by the fact that the yellow-coloured nucleoli it contains remain relatively small compared with those in the primary and vegetative nuclei. The generative nucleus stains yellow before it assumes a crescent-shape but it is impossible to say whether the change occurs at a stage of prophase corresponding to that observed in the meristematic cells. The chromosomes it contains continue to bind orange G for the remainder of the pollen development.

During the development of the pollen grain the cytoplasm which contains the vegetative nucleus shows an increased tendency to retain orange G. The sap in the nucleus of the "generative cell" is not stained by this mixture of dyes; there is no cytoplasm associated with this nucleus.

There is some indication that the pollen-grain wall has the capacity to stain yellow but this is difficult to determine from sections. The large cytoplasmic vacuoles which play an important role in maintaining correct orientation of mitosis in the pollen grain are always unstained.

DISCUSSION

Under certain conditions of fixation and pH the nucleoli and the chromosomes in their spiralized state stain yellow with orange G-aniline blue. Elsewhere it has been suggested that there is an intimate relationship between nucleolar material and chromosome matrix [1, 11]. This idea was based largely on the belief that the formation and loss of matrix coincide with the dissolution of the nucleoli at prophase and their re-formation at telophase. There are diverse opinions however as to the constitution of the matrix; some even doubt its existence and are particularly sceptical of the view that the spiralized chromosome is totally embedded in a matrical mass (cf. [4]). Earlier studies had led to the suggestion that there was a relationship between the matrix and the ribose nucleic acid (RNA) of the nucleolus [2]. This view was mainly founded on the supposition that counter-staining with light green after the Feulgen reaction demonstrated the sites of RNA, an assumption that has since been shown to be erroneous because the hydrolysis used in the reaction removes all the RNA from the cells. The suggestion has been revived, however, by recent evidence that mitotic chromosomes may contain RNA [5, 3].

From data to be presented in a subsequent paper it seems that the yellow reaction obtained on the nucleoli and chromosomes is associated with phospholipids. It might be contended that these must be two dissimilar lipids, one for each site, since it has been found that prophase chromosomes stain yellow while the nucleoli are still present. However, this observation could be interpreted as being caused by the presence of only one phospholipid which is transferred from the nucleolus to the prophase chromosomes, with the renewal of the nucleolar store of this lipid, that is by a turnover of the nucleolar phospholipid.

The spiralization of the chromosomes seems to coincide with the uptake of the yellow-reacting substance. There is no evidence, however, that it is responsible for spiralization, although there is clearly a correlation between the spiralization of the chromosomes and the occurrence of this substance as shown by its presence on the condensed heterochromatic regions at interphase.

According to some workers the "matrix" plays an important part in chromosome spiralization [6, 7 cf. [3]. Whatever the role of the phospholipid on the chromosome, it is clear that it is not a "matrix" in the sense that the fully spiralized chromosome is solidly embedded in it. Rather is it to be re-

garded as a coating to the nucleic acid–protein structure of the chromosomes, which is deposited about the time that spiralization begins.

SUMMARY

1. After the fixation of roots and anthers in Lewitsky's fluid the chromosomes in mitosis and meiosis show a cyclic staining behaviour in a mixture of orange G and aniline blue. In mitosis the change occurs before the dissolution of the nucleoli and in meiosis when the dissolution of the nucleoli is almost complete.

2. In root tips of seven species the euchromatic parts of chromosomes stain yellow from about mid-prophase to the end of anaphase but blue at telophase, interphase and early prophase. Heterochromatic regions, however, in species where they are detectable, stain yellow at all times except in early prophase.

3. A deviation from this staining behaviour is found in root tips in a narrow region of peripheral cells and in cells adjacent to the root cap. In these cells the chromosomes at all times stain yellow and the cytoplasm yellowish. A study of the early development of lateral roots in *Vicia faba* indicates that this is not an artefact.

4. Examination of pollen grains in two diploid species of *Tradescantia* show that during mitosis the chromosomes undergo changes in staining similar to those found in meristematic cells. The vegetative and generative nuclei which arise from this mitosis have different staining qualities. The vegetative nucleus stains blue for a long period of pollen development but ceases to stain at all just before anthesis; the generative nucleus, however, stains blue for a brief period and yellow thereafter.

5. In pollen-mother cells the chromosomes stain blue at leptotene and pachytene but yellow during diakinesis. At the second division the chromosomes stain as in mitosis.

6. Under these conditions of fixation and staining, nucleoli always stain yellow, the cytoplasm stains greyish, the spindle blue but the nuclear sap remains colourless. The cytoplasm in the "vegetative cell" in pollen grains has an increased tendency to bind orange G.

7. The staining behaviour of the chromosomes and nucleoli are briefly discussed in relation to earlier ideas on the chromosomal "matrix".

We should like to thank Dr. K. S. Dodds and Dr. Honor B. Fell, F.R.S., for their helpful criticism. We are also grateful to Professor J. T. Randall, F.R.S., for his interest and provision of working facilities in the Wheatstone Physics Laboratory, King's College.

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EVIDENCE FOR LIPID MATERIAL IN CHROMOSOMES

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Received August 22, 1957

A CYCLIC staining behaviour of the chromosomes during mitosis and meiosis was found when sections of root tips and anthers were stained in a mixture of orange G and aniline blue after fixation in Lewitsky's fixative [9].

The present paper is concerned largely with the histochemical basis for this differential staining behaviour.

MATERIALS AND METHODS

Root meristems from the following plants were studied: *Fritillaria falcata*, *Scilla sibirica*, *S. campanulata*, *Trillium grandiflorum*, *Paris polyphylla* and *Vicia faba*.

The following fixatives were used: Acetic alcohol (1:3); Carnoy (ethanol–chloroform–acetic acid 6:3:1, by volume); AFA (ethanol–formal–acetic acid, 30:15:1 by volume [14]); 45 per cent acetic acid; Lewitsky's fluid (1 per cent chromic acid and 10 per cent formalin in equal volumes [11]); weak Bouin [1]; formal–calcium [1]; Zenker with acetic acid and Helly's modified Zenker in which the acetic acid is replaced by formalin [8].

The root tips were embedded in paraffin wax by passing them through chloroform prior to infiltrating with wax [9]. Sections were cut at 7 μ.

The staining mixture was prepared by dissolving 2 g of orange G and 0.5 g aniline blue (wat. sol.) in 100 ml of citrate buffer (Hopkins and Williams) at pH 3; this was not very critical and pH might vary between 2.5 and 4. After removal of wax the sections were taken down to distilled water, stained for 2–3 minutes and then rinsed for at least 30 seconds in citrate buffer at the pH used for preparation of the stain. The sections were then carefully blotted, rinsed rapidly in equal parts of absolute ethanol and tertiary butanol, rinsed in tertiary butanol for 2–3 minutes and made permanent in Euparal.

The Effect of Fixation on Differential Staining

The differential staining of chromosomes, nucleoli and cytoplasm with orange G–aniline blue depends on the fixative employed. When root meristems are fixed in Lewitsky's fluid the chromosomes in interphase nuclei

TABLE I. *The effect of fixation on the staining of meristematic cells in roots with orange G–aniline blue.*

Fixative	Metaphase chromosomes	Nucleoli	Heterochromatin at interphase	Cytoplasm
45 % acetic acid	blue	blue	blue	blue
Acetic alcohol	blue	yellow	blue	bluish
Carnoy (6:3:1)	blue	yellow	blue	bluish
Lewitsky	yellow	yellow	yellow	blue-grey
Helly	blue	yellow	blue	blue-grey
Zenker	yellow	yellow	yellow	greyish
AFA	blue	yellow	blue	bluish
Formal–calcium	blue	yellow	blue	blue-grey

stain blue except in their heterochromatic parts which, like the nucleoli, stain yellow, but from mid-prophase until early telophase the chromosomes stain uniformly yellow. Throughout the mitotic cycle the cytoplasm is coloured blue-grey; the spindle stains blue but the nuclear sap remains colourless.

There are several fixatives, however, after the use of which the cyclic staining behaviour in the chromosomes is not found, the chromosomes staining blue at all phases of the mitotic cycle. On the other hand, the nucleoli stain yellow after a wide range of fixatives including those after which the chromosomes stain blue. A notable exception is 45 per cent acetic acid after which both the chromosomes and nucleoli stain blue. The nucleoli show an intermediate staining behaviour after long fixation in acetic alcohol; after fixation of roots for one hour the nucleoli stain a clear yellow, but with periods of from 16–24 hours they stain with both the aniline blue and orange G. The colouration of the cytoplasm also is influenced by the fixative used; after some it stains a bright blue with the double stain.

White [16] considered that although cytoplasmic proteins stain initially with orange G, this dye is later displaced by aniline blue when the two dyes are used competitively. Various times of staining were therefore tried. The characteristic staining results were unaltered if the tissue was stained for from 30 seconds to 5 minutes, although after longer times of treatment there was an increased tendency for the yellow stain to become masked by that of the aniline blue. The characteristic colouration of metaphase chromosomes, nucleoli, heterochromatic regions and cytoplasm after a number of fixatives is presented in Table I.

The Effect of Ribonuclease Treatment

The staining behaviour found in mitotic cells after fixation in Lewitsky's fluid, is not due to the presence or absence of desoxyribose nucleic acid (DNA), and probably not to the selective staining of DNA and ribose nucleic acid (RNA) in the cells. RNA is found both in the nucleoli and in the cytoplasm but these react in opposite ways to the mixture of stains. Since, however, it was found that the nucleoli stain yellow after most fixatives it was thought necessary to examine further the possibility that the yellow colouration might be due to RNA.

Roots were therefore fixed for one hour in acetic alcohol, hardened in absolute ethanol, embedded and sectioned. About 12 sections were placed on each slide, and the slides were treated in pairs. The wax was removed, the preparations were taken down to water and the sections on each slide were separated into three groups by applying melted paraffin wax with a heated wire so as to form a narrow wax wall, 2–3 mm high, round them care being taken to make an efficient wall between each group. The compartment so formed served to carry ribonuclease in one, buffer in another and distilled water in the third. The nuclease (Worthington) was dissolved in a 0.1 *M* HCl–veronal buffer of pH 7.7 and the same buffer alone was used in the compartment provided for it on each slide. The nuclease was allowed to act for one hour. The slides were then rinsed thoroughly in distilled water and the wax walls removed. One slide was stained in methyl green pyronin for 30 minutes, the other for 3 minutes in orange G–aniline blue.

After methyl green–pyronin staining the nucleoli and cytoplasm were devoid of stain in the sections which had been treated with ribonuclease in contrast with their uptake of pyronin in the control sections on the same slide. On the slide which had been stained with the orange G–aniline blue, however, the treated and control sections were indistinguishable; the nucleoli in both stained yellow. It can therefore be assumed that the yellow colouration of the nucleoli is not due to RNA. We assume that this is true also of the yellow reaction obtained in the metaphase chromosomes when stained after fixation in Lewitsky's fluid. Ribonuclease cannot be used after this fixative which inhibits its activity, but short hydrolysis in *N*/HCl enhances the yellow colour; the reverse would be expected if the yellow reaction were due to RNA.

The Staining of Known Substances with Orange G–Aniline Blue

It was clearly of interest to discover the cause of the strong retention of orange G by nucleoli or chromosomes after the various fixations. It was

possible that this phenomenon was associated with the presence of a particular molecule and hence a range of substances was tested. This included simple proteins such as Bovine plasma albumen, β-lacta globulin, egg albumen, collagen and histone; a phosphoprotein; desoxyribose nucleoprotein, precipitated by salt; RNA (yeast nucleic acid dissolved in water and shaken with chloroform to remove impurities); folic acid; fatty acids; ergosterol; a crude sample of egg-lecithin and pure samples of phosphatidyl choline and phosphatidyl ethanolamine. Drops of the solutions of the proteins and of the RNA were filmed thinly on slides and dried; a coating of Meyer's albumin was necessary for the RNA to prevent its becoming washed off the slides subsequently. The nucleo-protein was smeared on to slides and dried. The folic acid was dissolved in methanol and drops of the solution were dried on to slides. The lipids were dissolved in chloroform and drops of these solutions were dried on to slides. The fatty acids were absorbed in Whatman filter paper or on cigarette paper. All the material on slides was fixed for one hour in Lewitsky's fluid, washed for 15 minutes and rinsed in citrate buffer prior to staining.

The colour reactions obtained are recorded in Table II. No reaction was given by the fatty acids because the stain was repelled by them. Only four substances stained yellow, namely crudely-prepared histone, desoxyribonucleo-protein, phosphatidyl choline and egg-lecithin. Purified samples of the first two substances stained blue, suggesting that the yellow colour was due to an impurity. When films of the impure samples, as well as lecithin, were fixed with formal–calcium, however, they stained blue and there was a strong tendency for the lecithin to go into colloidal solution.

A Comparison between the Response to the Double Stain and General Lipid Tests

Since the egg-lecithin gave a yellow reaction when stained in orange G–aniline blue, it was thought desirable to compare the response of tissue to this mixture of dyes with that found for lipid stains such as Sudan black, Nile blue sulphate and acid haematein.

Sudan black is a fat-soluble dye and has been widely used as a general fat stain (cf. [4]). Nile blue sulphate is alleged to combine readily with fatty acids and under suitable conditions is said to distinguish between neutral lipids which stain red and acidic lipids which stain blue [3, 4]. The acid haematein method is considered by Baker and Cain [1, 3, 4] to be specific for phospholipids provided that the pyridine extracted controls give negative

TABLE II. *Staining behaviour of known biological substances with orange G–aniline blue.*

Proteins	
Bovine plasma albumen (0.1 % solution)	blue
β-lacta globulin (Armours, 0.1 % solution)	blue
Histone (from calf thymus, *crude* preparation)	yellow
Histone (from calf thymus, *purified* preparation)	blue
Collagen	blue
Albumen (Meyer's adhesive for paraffin sections)	blue
Phosphoprotein	
Casein (prepared by McMeekin, U.S.D.A.)	blue
Nucleoprotein	
DNA–histone (from calf thymus, *crude* preparation)	yellow
DNA–histone (from calf thymus, *purified* preparation)	blue
Ribose nucleic acid (B.D.H. sodium salt)	blue
Folic acid (Lights)	blue
Phospholipids	
Lecithin–egg (B.D.H. used after chloroform extraction and repeated acetone precipitation)	yellow
Lecithin–egg DL α,β-dipalmitoyl (phosphatidyl choline, prepared by T. H. Bevan)	yellow
Cephalin, α,α'-dimyristil (phosphatidyl ethanolamine, prepared by T. H. Bevan)	blue
Fatty acids	
Oleic acid (B.D.H.)	no reaction
Linoleic acid (B.D.H.)	no reaction
Sterol	
Ergosterol (B.D.H.)	blue

results. According to these authors pyridine is the most satisfactory solvent for the removal of lipids from fixed tissues.

The tests were made on sections of root tips fixed in different fixatives and given various treatments, viz. fixation in Lewitsky's fluid, formal–calcium, acetic alcohol, 45 per cent acetic acid or weak Bouin followed by the pyridine extraction test of Baker. Some of the roots fixed in acetic alcohol were removed from the fixative after 16 hours and immersed for 5 minutes in boiling ethanol–ether; this was repeated three times, a fresh solution being used each time.

TABLE III. *The effect of fixation and extraction on the staining response of nucleoli and chromosomes to lipid stains and orange G–aniline blue.*

pos. = positive; neg. = negative; int. = intense; spor. = sporadic.

	SUDAN BLACK		NILE BLUE		ACID HAEMATEIN		ORANGE G–ANILINE BLUE	
	Nucleoli	Met. chrom.	Nucleoli	Met. chrom.	Nucleoli	Met. chrom.	Nucleoli	Met. chrom.
Lewitsky	neg.	neg.	int. blue	int. blue	pos.	pos.	yellow	yellow
Formal–calcium plus pre-mordant	weak	neg.	blue	neg.	pos.	neg.	yellow	blue
W. Bouin, pyridine plus pre-mordant	int.	spor.	blue	spor.	mostly pos.	spor.	some blue ,, yellow	yellowish
Acetic alcohol	int.	weak	blue	weak blue	pos.	neg.	yellow	blue
Acetic alcohol, ethanol ether	int.	—	blue	blue	pos.	—	pale yellow	—
45 % acetic acid	int.	weak	neg.	blue	neg.	neg.	blue	blue

In order to apply the acid haematein reaction after Lewitsky's fluid the roots were fixed for 18 hours; mordanting of the tissues in dichromate was omitted since the fixative itself contains chromium. When other fixatives were employed, the roots were fixed for 6 hours and then treated with dichromate for 18 hours at room temperature. At first, Baker's schedule for staining and differentiation was followed, but in another series of tests the recommended time in hot dichromate was reduced for sections of tissues fixed in Lewitsky's fluid. The Nile blue sulphate was used at two concentrations, as recommended by Cain [3].

The results obtained were somewhat confusing, particularly in regard to the staining behaviour of nucleoli (Table III). The material fixed in Lewitsky's fluid was exceptional in that the nucleoli were unstained with Sudan black, but nevertheless gave an intense blue colouration with Nile blue at both concentrations, and a reaction after acid haematein. Seemingly contradictory results were also obtained in nucleoli after fixation in 45 per cent acetic acid. An intense reaction was obtained with Sudan black, but a negative result with acid haematein and with Nile blue at both concentrations.

The nucleoli gave reactions with all three lipid tests after formal–calcium but with Sudan black the staining was very weak.

The treatments with ethanol–ether and pyridine were disappointing. As judged by the reactivity of the nucleoli it is evident that ethanol–ether removes little if any lipid material from these sites. The pyridine however led to variable staining of the nucleoli both by the acid haematein and by orange G–aniline blue. Sporadic reactions were obtained with the former method and with the latter the nucleoli sometimes stained blue and sometimes yellow.

The treatment with pyridine also led to the metaphase chromosomes and interphase nuclei sometimes staining yellow with the double stain, and was similarly responsible for their giving occasional reactions with the lipid stains.

The metaphase chromosomes preserved in Lewitsky's fluid gave reactions with acid haematein and with Nile blue at both concentrations, but not with Sudan black. In this they resembled the nucleoli after this fixation. After the other fixatives (Table III) no response to the lipid stains was shown by metaphase chromosomes except after acetic alcohol where a weak blue reaction was obtained in the strong concentration of Nile blue.

From the results (Table III) it will be seen that the reactions of the cell components to the acid haematein and orange G-aniline blue corresopond in that wherever a positive reaction is obtained by the first a yellow reaction is given by the second. One exception to this is in the euchromatin at interphase which is coloured strongly by the acid haematein method but stains blue with the orange G–aniline blue mixture (Figs. 3, 8). This discrepancy can be removed if the length of treatment with the hot mordant, usually 24 hours, is shortened to e.g. 5 hours (Figs. 1 2, 4 and 5). Under these conditions, absence of a blue-black reaction in the euchromatic regions of the nucleus cannot be construed as absence of phospholipid; all that might be concluded is that there is some difference between the material of the euchromatic and heterochromatic regions. That this might be a qualitative difference is indicated by the fact that after fixation in Lewitsky's fluid the euchromatic regions stain brown with the haematein although the heterochromatic regions remain intensely blue-black. It is very likely that there is a quantitative difference in the two regions also. Whether the reduced time in hot dichromate mordant used for sections of tissues fixed in Lewitsky's fluid is a valid modification can be decided only by a complete series of tests of the kind made by Baker [1].

The discrepancies in behaviour of other lipid stains are discussed later.

The Extraction of Lipoidal Substances by Fixation

The cytological results suggested that fixation in acetic alcohol, acetic acid or formal–calcium resulted in loss of lipoidal matter from root tip cells, although Lewitsky's fluid apparently preserved this material inside the cells. If this is correct, the fatty substances lost from the tissue should be demonstrable in fixing solutions of the first type after use, but not in the used Lewitsky's fixative.

To test this, fifty root tips, about 1 cm in length, of *Trillium grandiflorum* were fixed for 6 hours in 5 ml formal–calcium and another fifty in the same volume of Lewitsky's fluid for the same time. The roots from the former were transferred to the dichromate mordant for 18 hours while those from the latter were immersed in fresh fixative for 12 hours. Both samples were embedded in wax and sections cut and stained to confirm that the results were normal, i.e. as shown in Table I.

The solutions in which the roots had been fixed were filtered and shaken with an equal volume of chloroform. A heavy milky precipitate formed at the interface with the formal–calcium but no precipitate was produced from Lewitsky's fluid. The portion including the interface and the chloroform was removed and the formal–calcium was shaken with fresh chloroform; the process was repeated until no further precipitate formed. Similar results were obtained when the used fixing solutions were shaken with a solution of Reinecke's salt in methanol [15].

The precipitate was dried on to slides and examined. It appeared to consist of two components, one fibrous and the other fatty. Such smears were fixed in Lewitsky's fluid for 15–30 min., which rendered them insoluble in subsequent treatments. They stained with Sudan black and with the acid haematein technique and yielded a predominantly yellow colour with the orange G aniline blue test. It was clear that the reactions which were lacking in the metaphase chromosomes and in the heterochromatic regions of the nuclei after fixation in formal–calcium, were present in the precipitate

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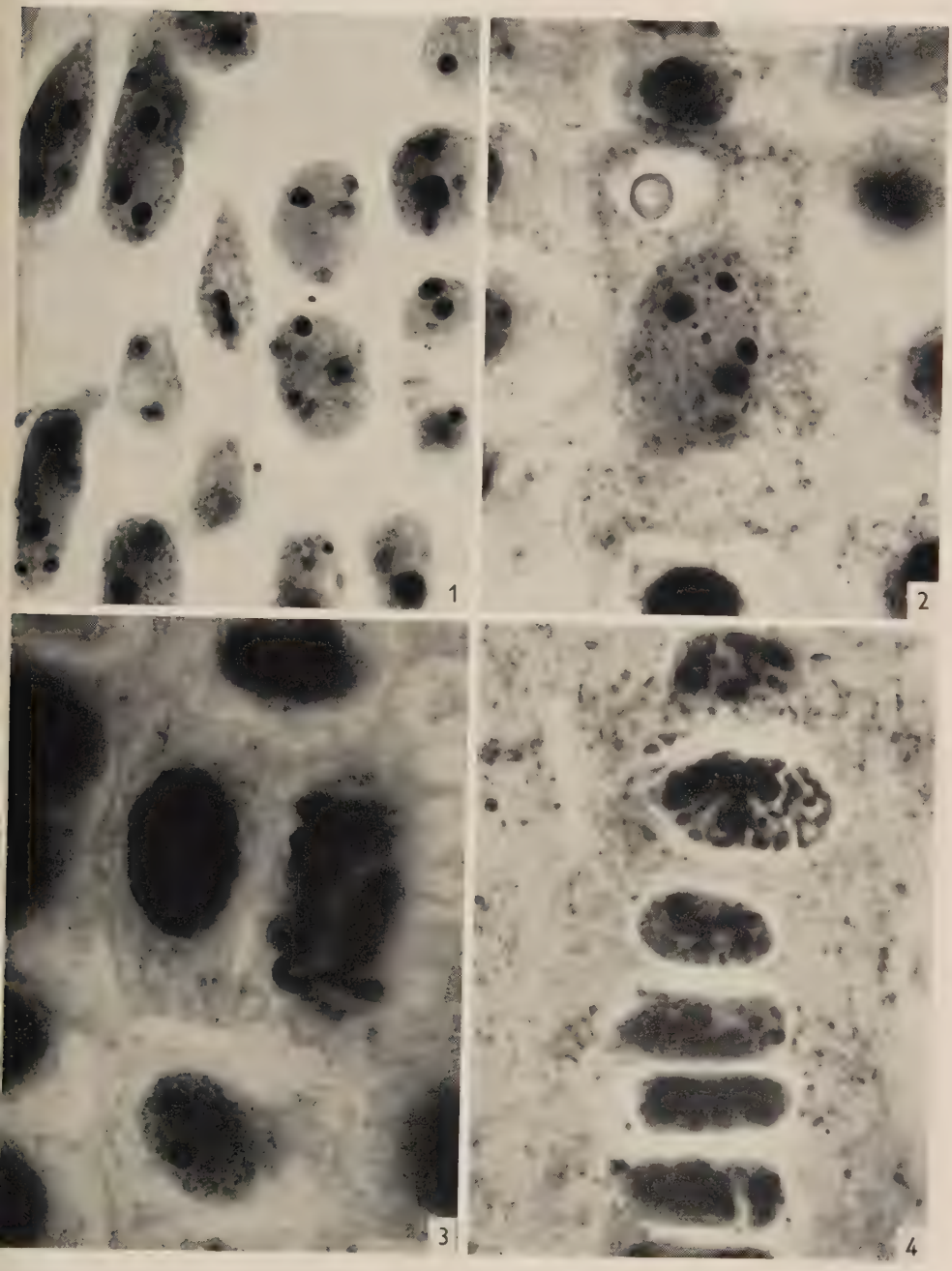
Figs.1–4.—Microphotographs of meristematic cells in roots from sections stained with acid haematein. Figs. 1–3 from *Trillium grandiflorum;* Fig. 4 from *Fritillaria falcata.* Figs. 1, 2 and 4 taken with a tricolour red filter. Fig. 1 after fixation in formal–calcium; Figs. 2, 3 and 4 after fixation in Lewitsky's fluid. The sections in Figs. 1 and 3 received hot dichromate mordant for 24 hours; those in Figs. 2 and 4, for 5 hours. Note the negative response of the chromosomes during interphase and prophase in Fig. 1; the positive reaction of the heterochromatic regions during interphase in Figs. 2 and 4; the positive reaction of the prophase chromosomes in the latter and the uniform reaction of the chromosomes during interphase and mitosis after receiving the full period of dichromate mordanting in Fig. 3. All figures × 1000.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which had been extracted from the tissues by this fixative. The precipitate gave a strongly positive reaction with Sakaguchi test for arginine as modified by McLeish *et al.* [12]; this implied that some protein-like material might also have been removed from the roots by the fixative. This was surprising in view of the fact that formalin is considered an excellent fluid for preserving protein in tissues. A detailed analysis of the matter extracted from plant roots by formal calcium, and other fixatives is in progress, but for the present study it was obviously important to demonstrate that this precipiate which gave positive results with cytological reactions for lipids, actually contained these substances. Two hundred and fifty root tips of *Trillium grandiflorum* were therefore fixed in formal–calcium for 6 hours. The fixing solution was then filtered and shaken with chloroform, as described above, and the precipitate was collected and dried. Some roots were embedded, sectioned and stained as described previously, in order to check the normality of the roots and the efficiency of the extraction of the suspected phospholipid. The dry precipitate was heated in boiling alcoholic potash ($N/2$ potassium hydroxide in 95 per cent ethanol) for 2 hours in a flask equipped with a long glass-tube to act as an air-cooled reflux condenser. After cooling, saponifiable and unsaponifiable fatty matter were removed by shaking with ether, and the water-soluble material was concentrated and examined by ascending partition chromatography on paper, with 80 per cent propanol as the solvent. Although no appreciable amounts of choline, serine or ethanolamine have been found, the presence of α-glycerophosphate has been demonstrated clearly and repeatedly in such experiments. These substances were visualised as follows: serine and ethanolamine by spraying with a solution of ninhydrin in butanol; choline by the method of Levene and Chargaff [10] and glycerophosphate by that of Hawes and Isherwood [7] *cf.* [2].

Staining Tests after Fixation in Fluids Containing Reinecke's Salt

It will be recalled that Reinecke's salt can be used to separate lipid material from the used formal–calcium fixative. An experiment was therefore made

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Figs. 5–8.—Microphotographs of meristematic cells in roots, Figs. 5 and 6 from *Scillia sibirica*, Fig. 7 from *Scilla campanulata* and Fig. 8 from *Fritillaria falcata*. All taken with a tricolour red filter. Figs. 5 and 6 after acid haematein (5 hours in hot dichromate mordant); Figs. 7 and 8 after staining with orange G -aniline blue. Figs. 5 and 8 after fixation in Lewitsky's fluid; Figs. 6 and 7 after fixation in formal–calcium plus Reinecke's salt. Note the closely similar staining behaviour in Figs. 5 and 6 and the difference in staining behaviour of the chromosomes during interphase and mitosis in Fig. 7. In Fig. 8 note the divergent response of the eu- and hetero- chromatic regions in interphase nuclei; the yellow-stained heterochromatic regions appear as lightly stained areas and are almost indistinguishable from the nucleoli. All figures × 1000.

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to see whether this substance would precipitate the lipid material *in situ* if combined with formal–calcium as a fixative.

Roots of *Scilla campanulata* and *S. sibirica* were fixed in formal–calcium plus Reinecke's salt (0.05 g in 5 ml of fixative). Some of the roots were fixed for 18 hours and others were transferred after 6 hours to the dichromate mordant where they remained for 18 hours. The roots were then embedded and sections were mounted on slides together with sections of material fixed in formal–calcium alone, to serve as a control. For the acid haematein test, some slides of the material which had received dichromate treatment were mordanted in dichromate at 60°C for 24 hours and some for only 5 hours. The orange G–aniline blue mixture was used on sections which received no dichromate treatment.

Formal–calcium fixation in the presence of Reinecke's salt, gave results identical with those obtained after Lewitsky's fluid, both with the acid haematein and the orange G aniline blue methods (Figs. 6 and 7). Thus after the longer treatment in the mordant the chromosomes gave positive results in the acid haematein at all phases of the mitotic cycle while after the shorter treatment, only those parts stained that were in a spiralized state.

Reinecke's salt is prepared by the fusion of ammonium thiocyanate and ammonium dichromate [13]. Neither of these compounds when added individually to formal–calcium have the same effect as the Reinecke's salt. The fixation is poor and there is a strong suggestion of a diffusion of substances in the cells. When sections were stained in orange G–aniline blue some interphase nuclei were blue and some yellow. After ammonium dichromate the nucleoli stained yellow but at different intensities some being almost colourless.

Some roots were fixed in absolute methanol containing the same amount of Reinecke's salt and some in methanol alone. The roots were embedded, sectioned and stained with the orange G–aniline blue mixture. After methanol alone the chromosomes stained blue and the nucleoli a bluish-yellow. With the addition of the salt some interphase nuclei stained blue and contained yellow-coloured nucleoli; others of a similar stage of interphase stained partly or wholly yellow. The clearest indication of diffusion of material, however, was found in certain cells which contained yellow-staining masses, all similarly orientated, at the innermost walls of the cells, often partly engulfing the nucleus. The diffusion of this material, which we conclude to be lipid, is probably due to the more rapid penetration of the methanol than of the Reinecke's salt.

DISCUSSION

The identification of phospholipid.—Baker's acid haematein and pyridine extraction tests for phospholipid [12] can be used to demonstrate the presence of phospholipid in a region of a cell if (*a*) it stains blue-black after the treatment described by Baker, which includes fixation in formal–calcium, and (*b*) it does not stain after fixation in dilute Bouin's fluid and extraction in hot pyridine. Baker recognised that some parts of cells retain the ability to stain after these tests and that, moreover, some will stain only after treatment with pyridine. He was not unaware of the possibility of "masked lipid" being present, but the considered that phospholipid can be identified with a reasonable degree of certainty only where the acid heamatein reaction gives a positive result and the pyridine extraction test is negative. It is not suggested that where both are positive there is no phospholipid present; it is argued only that there is no proof unless the material, suspected of being lipid by the first test, can be extracted by a fat solvent, such as pyridine.

In the present study an immediate difficulty was that the chromatin did not stain with the acid haematein method after fixation in formal–calcium, although it reacted strongly after fixation in Lewitsky's fluid. Furthermore after roots had been fixed in these fluids, material which gave this reaction could be precipitated from formal–calcium but not from Lewitsky's fluid. This implied that the cells of the roots investigated contained a substance which might be a phospholipid in view of its staining behaviour and which was fixed in the cells by Lewitsky's solution but was extracted from them by formal–calcium. The proof of that this particular substance was truly a phospholipid depended on its analysis and this was made possible by the development in recent years of elegant procedures for the identification of small amounts of phospholipids by partition chromatography. The material extracted from roots of *Trillium grandiflorum* by formal–calcium and precipitated by shaking with chloroform was therefore subjected to these tests. As was to be expected, the formal–calcium did not remove lecithin or cephalin in that no appreciable amounts of choline, serine or ethanolamine were identified in the hydrolysate. The presence of α-glycerophosphate, however, is taken as proof that the substance extracted was a phospholipid, and hence as confirmation of the result of the acid haematein test. On the present evidence, it would seem to be an unusual phospholipid of the lecithin type, but containing an exceptional base which was not identified by the tests used in this investigation. This problem is being studied further.

In comparing the results obtained by fixing tissues in Lewitsky's fluid and

in formal–calcium, the following conclusions seem justified: with the former fixative, the chromatin may be stained by the acid haematein method, and by orange G when the dye mixture is used, and no lipid can be identified in the fixative. With the latter, the chromatin does not stain with acid haematein or orange G, but material that gives these staining reactions is extracted from the cells by the fixative; such material has been shown to contain a phospholipid.

The histological demonstration of phospholipid.—It seems clear, therefore, than an unusual form of phospholipid is present in roots of *Trillium grandiflorum*, which is extracted from the tissues by formal–calcium but is fixed in them by Lewitsky's fluid. Histochemical data suggest that such a lipid occurs in the roots of other plants. After fixation in Lewitsky's fluid the phospholipid is situated exclusively on the chromosomes, as shown by staining with the acid haematein and the orange G–aniline blue methods. In the present study, these procedures are taken to indicate the presence of phospholipid because they stain both the extracted matter which contains this substance and cells in which the substance occurs, but they do not stain the cells from which it has been extracted. Moreover the results with the acid haematein tests are paralleled by those with orange G–aniline blue and it has been shown that of the substances tested, only lecithin retained the yellow colour with the dye mixture. It is not claimed that only lecithin stains thus; the test material was crude and it is possible that the reaction is given by some material associated with phospholipids rather than by these compounds alone.

The precise localisation of the phospholipid.—Ideally cytochemical studies should be made on tissues which are fixed in a life-like conditon, so that there can be no danger of the fixation destroying the sites of the substance investigated or rendering it soluble and allowing it to diffuse about the cell organelles. Fixation in methanol, or in formal–calcium renders the phospholipid soluble and removes it from the cell. The addition of Reinecke's salt to methanol results in clear signs of diffusion of the lipid and of its non-specific precipitation in the cell. When this salt is added to formal–calcium, however, the phospholipid is found at specific sites only, which are identical with those which bear this lipid after fixation on Lewitsky's fluid. If this were due to the phospholipid being freed from its primary site, diffusing about the cell and then becoming adsorbed on to the chromosomes, it is difficult to understand (*a*) why this does not occur in formal–calcium alone and (*b*) if the adsorption on to the chromosomes is aided by the presence of Reinecke's salt, why this substance does not have the same effect when

added to methanol. Hence, although precise proof is lacking, there would seem to be a strong case for presuming that the localisation of this phospholipid after fixation in Lewitsky's fluid and in formal calcium Reineckeate is not an artefact.

Another problem is whether, at telophase and interphase, this phospholipid is present on the heterochromatic regions alone, or also on the euchromatic parts. The unmodified acid haematein method shows it on all parts of the chromosomes, but the modified method and the orange G–aniline blue stain suggest that it occurs only on the heterochromatic regions. The fact that the modified technique involving shortening of the time in the hot mordant, stains the euchromatic parts brown, not black, might be taken to indicate some qualitative difference between the lipids of these two parts of the chromosomes. This view is supported by the very striking results with the mixture of dyes: the euchromatic parts are stained blue and the heterochromatic parts yellow [9]. Complete proof of this qualitative difference must await the discovery of the precise action of this dye mixture, but the evidence at present available suggests that a phospholipid becomes associated with the chromosomes from about mid-prophase to the end of anaphase and thereafter is lost from them, except in those regions which remain spiralized, namely the heterochromatic parts.

The staining of nucleoli.—That the nucleoli may contain phospholipid is suggested by their staining with the acid haematein method after formal–calcium fixation and with orange G in the dye mixture, whereas after fixation in 45 per cent acetic acid, which acts as a very efficient solvent for lipids in these cells, the nucleoli do not react with these tests. The results are not conclusive, however, since even after treatment in 45 per cent acetic acid, the nucleoli are coloured with Sudan black. It is possible that this is due to the Sudan black acting as a basic dye as suggested by Casselman [5] or to the presence in the nucleoli of some other lipid which is not extracted by 45 per cent acetic acid. Nucleoli are unstained by Sudan black when fixed in Lewitsky's fluid, but this might be due to the inability of the phospholipid to adsorb this colouring agent after this treatment owing, for example, to the deposition of a protective surface film of protein on the lipid. It seems clear, however, that if nucleoli contain a phospholipid, it is either different from that found on the chromosomes, or is more tightly bound, in that it is not extracted by formal–calcium nor by some other fixatives (see Table I). Like the results with Sudan black, those with Nile blue are difficult to explain completely except on the basis that this dye-complex too is acting, at least partially, as a basic dye.

SUMMARY

1. A histochemical investigation has been made of a differential staining behaviour observed in chromosomes when sections of roots are stained in a mixture of orange G–aniline blue.

2. The staining behaviour is dependent on the type of fixation. After Lewitsky's fixative the chromosomes stain uniformly yellow from about mid-prophase until the end of anaphase, but blue during telophase and interphase except in heterochromatic regions which stain yellow. After some other fixatives, e.g. acetic alcohol or formal-calcium, the chromosomes stain uniformly blue at all times. The nucleoli on the other hand stain yellow after these and most other fixatives.

3. A closely parallel staining behaviour was found when sections of roots were treated with Baker's acid haematein test for phospholipids, indicating that the yellow staining of the chromosomes with orange G–aniline blue was associated with the presence of phospholipid.

4. Material shown to be lipid has been recovered from formal–calcium solution used for the fixation of root tips but not from similarly used Lewitsky's fluid. Smears of this material, when fixed in Lewitsky's fluid, stain with Sudan black, give a positive acid haematein reaction and stain yellow with orange G–aniline blue. Arginine was also found in the smears as shown by the Sakaguchi test.

5. Biochemical confirmation of the presence of phospholipid in used formal-calcium solution was provided by means of partition chromatography which indicated that a substance which yielded α-glycerophosphate on hydrolysis was removed from the root cells by this fixative.

6. The staining behaviour of the nucleoli in relation to that of the chromosomes, and some problems connected with the acid haematein test and other lipid stains are discussed briefly.

We should like to thank Dr. K. S. Dodds and Dr. Honor B. Fell, F.R.S., for their helpful criticism. We are also grateful to Professor J. T. Randall, F.R.S., for his interest and provison of working facilities in the Wheatstone Physics Laboratory, King's College.

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SH COMPOUNDS IN MITOSIS

I. THE ACTION OF MERCAPTOETHANOL ON THE EGGS OF THE SAND DOLLAR *DENDRASTER EXCENTRICUS*

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Received August 23, 1957

This study was designed to test a prediction that follows from the hypothesis that the assembly of the mitotic spindle and asters from the protein molecules of which they are composed involves the thiol groups of the proteins [9, 10].

The argument of the experiment was as follows. If the formation of the mitotic apparatus involves the establishment of disulfide bonds (or other bonds involving intermolecular links through S) then it should be prevented or even reversed by introducing into the system an appropriate SH compound in sufficiently high concentration. If the mechanism of protein-to-protein linkage involves the oxidation of SH, the added compound should prevent this by maintaining reducing conditions. If it involves another type of linkage involving SH, the added SH compound should compete with the protein SH for the bonding sites, and thus prevent or reverse the polymerization. The reagent chosen was mercaptoethanol (monothioethylene glycol).[3] This reagent has been recommended for work on S–S bonds in keratins and other proteins [12] because it is conveniently neutral and is not very susceptible to autoxidation. On the basis of its structure, one might predict that it should not be very toxic, and this proved to be the case. A low toxicity for cells has been shown in investigations where it has been used in high concentrations in radiation-protection experiments [6]. It was found that mercaptoethanol penetrated the cells used in our studies readily, as would be predicted from its structural analogies to ethanol and glycol. It was anticipated that the predicted effect—a blockage or reversal of the formation

[1] Permanent address: Department of Zoology, University of California, Berkeley. This work was performed during residence as visiting professor at the Scripps Institution of Oceanography. Dr. Roger Revelle, the Director of that institution, and the many members of its staff who assisted me in innumerable ways will, I trust, appreciate the inadequacy of a footnote for the expression of my gratitude. This work was also assisted by grants from the American Cancer Society and from the Office of Naval Research.

[2] Contributions from the Scripps Institution of Oceanography, New Series, No. 961.

[3] I wish to thank my colleague Professor Harold Tarver for introducing me to mercaptoethanol.

of the mitotic apparatus—would take place only at quite high intracellular concentrations, sufficient to overwhelm the action of glutathione and other SH compounds already present.

EXPERIMENTAL

Most of the experiments were performed on the eggs of the sand dollar *Dendraster excentricus*, obtained by aqualung divers from the ocean bottom off the Scripps Institution of Oceanography, La Jolla, California, or from the bottom of Mission Bay, San Diego. I wish to thank Mr. James S. Kittredge, Mr. Raymond J. Ghelardi, Mr. Conrad Limbaugh and Dr. E. W. Fager for providing the living material and Professors A. Buzzati-Traverso and Denis L. Fox for making experimental facilities available. The eggs and sperm were obtained by injecting a small amount of 0.5 *M* KCl into the oral opening of the sand dollars, which induced shedding. The eggs were fertilized, washed free from excess sperm, and permitted to proceed toward cleavage. At the desired time, they were mixed with a stock solution of mercaptoethanol in sea water, generally freshly made, to the required final concentration. The experiments were carried out at 24°C at which temperature the time from fertilization to first cleavage is about 50 minutes.

OBSERVATIONS

Effect of Mercaptoethanol When Applied after Fertilization

Blockage of first cleavage.—In these experiments, designed to observe whether division can be blocked reversibly by mercaptoethanol, the *Dendraster* eggs were exposed to the reagent at 12 minutes after fertilization, and permitted to remain there for two hours and 15 minutes. After this long exposure, they were washed in sea water, and the reversal of the effect was observed. The results are summarized in Table I.

When the eggs were blocked in the one-cell stage for a long time, as in this group of experiments, they commonly divided directly into four cells when the mercaptoethanol was removed. Similarly, if they were blocked in the two-cell stage for several hours by a lower concentration of mercaptoethanol, they might proceed directly to the eight-cell stage when restored to their normal environment. This suggests that some reproductive processes, for example chromosome reproduction and the multiplication of centrioles, might have proceeded while the block was in effect.

A series of control experiments was set up in which identical concentrations of ethanol and mercaptoethanol were compared. There was no observable effect of ethanol at these concentrations. On the basis of the above findings, 0.1 *M* was the concentration chosen for most of the detailed studies of the effect of mercaptoethanol on division.

TABLE I. *Blockage of first cleavage by mercaptoethanol.*

Concentration *M*	Effect	Reversal
0.2	first cleavage blocked	begin division in 30 minutes after removal of mercaptoethanol
0.1	first cleavage blocked	go directly to 4-cell stage in 30 minutes
0.05	complete first cleavage blocked in 2-cell stage	go directly to 8-cell stage in 30 minutes

TABLE II. *Relation between mercaptoethanol concentration and number of divisions that can take place before blockage.*

Concentration *M*	Time from fertilization to 50 % first cleavage	Time from fertilization to 50 % second cleavage	Stage reached in 8 hours
0 (Control)	45	65	beginning gastrulation
0.005	47	73	early blastula not hatched
0.01	47	73	stopped at 16-cell stage
0.025	50	~85	stopped at 4-cell stage
0.05	54	blocked at 2-cell stage	

Effects of concentrations that do not block the first cleavage.—The blockage of division by mercaptoethanol could be an all-or-none effect, or could be a cumulative one. The fact that 0.05 *M* solutions permit the first cleavage but prevent the second favors the second alternative. This question was studied more closely by following the effects of a series of concentrations between 0.05 and 0.005 *M* applied eight minutes after fertilization. The results are given in Table II. The effect of mercaptoethanol on division is not an all-or-none effect in the sense that concentrations ineffective against the first division are without effect on later divisions. On the contrary, as is seen in Table II, the effect seems to be progressive in such a way that the number of divisions that is possible before blockage stands in a rough inverse relationship to the concentration of the agent. Even the lowest concentration (0.005 *M*) ultimately blocked further cleavage.

If, however, we consider the possible effect of mercaptoethanol on the aggregate of *rates* of processes involved in a given division, as measured by the time required to complete a division, an all-or-none or threshold relationship seems to appear. If a given concentration permits division to take place at all, it does not delay it greatly. These quantitative aspects of the mercaptoethanol effect will be considered in detail in another report.

The Stage at Which Mercaptoethanol Blocks Division

The *Dendraster* eggs were placed in 0.1 *M* mercaptoethanol at various times after fertilization, in order to observe whether there was a definite stage at which blockage was effective. The results are given in Table III.

It is obvious that there is a sharply definable stage in the progress toward division before which the blockage is completely effective and beyond which division proceeds normally in the presence of the mercaptoethanol. There was no evidence of a substantial delay of cleavage in the case of the samples that did cleave in the presence of the mercaptoethanol. This point, however, requires closer examination, because the progress of normal cleavage after 40 minutes is so rapid that a small delay might be significant of some effect on the cleavage process itself. The rise in the number of dividing cells between 34 and 38 minutes, and between 40 and 42 minutes is very striking and leads to the conclusion that there is a sharp transition from the stage at which mitosis is fully sensitive to the mercaptoethanol to that at which it is insensitive. An absolute discontinuity is not to be expected, since the synchrony of division is not perfect even in the *Dendraster* egg. Indeed, a spread of eight minutes between the time when the first 10 per cent of the population has passed a given stage and the time when the whole population has passed it is considered to be indicative of a very rapid transition from full sensitivity to full insensitivity.

It is not difficult to identify roughly the stage at which the transition takes

TABLE III. *The time of blockage of division by* 0.1 *M mercaptoethanol.*

Time after fertilization at which mercaptoethanol was introduced (min.)	Observations at 105 min. after fertilization
19	no division
24	no division
28	no division
32	no division
36	18 % 2-cell stage
40 (cells elongating)	80 % 2-cell stage
44 (early furrowing)	95 + % 2-cell stage
48 (furrowing)	95 + % 2-cell stage
Controls	8-cell stage, going to 16-cell stage

place in terms of the processes leading to cell division. The transition takes place somewhere in the brief period during which the metaphase configuration of the spindle is established and chromosome movement begins. What is blocked is either the final stages in the achievement of metaphase or the initiation of anaphase movement. Full blockage occurs even if the eggs are within a few minutes of the transition point. Examination of the living cells at the time when they have just reached the transition point shows that they have fully established spindles and are beginning to elongate. On the other hand, the blocked cells do not resemble normal metaphase cells, as might be expected if the effect was merely to arrest progress at that stage. One sees a rather large clear area within the cell, rather than the diastral configuration. This is true even of the cells that are observed to be at the metaphase stage at the time when the mercaptoethanol is applied. What is suggested visually is that we are not dealing with a mere arrest at this time, but rather with a situation in which the mitotic apparatus is modified structurally if it is not permitted to proceed through metaphase and anaphase. Observations on the isolated mitotic apparatus of the blocked cell reveal that it is indeed abnormal, and these findings will be presented in another communication.

Reversal of the Mercaptoethanol Effect

If the egg is arrested at the stage it is in when the mercaptoethanol is added, then the time from fertilization to division should be the normal time plus the period of exposure to mercaptoethanol. If, on the other hand, the progress toward division proceeds up to a sensitive stage, where the block occurs, then the recovery time should be relatively constant and independent of the stage at which the cells were exposed to the chemical. Here the work of Dustin [5] on the effect of dimercaptopropanol (BAL) on mitosis in mouse bone marrow may be relevant. He observed an accumulation of cells in metaphase; the cells apparently could proceed to that stage before they were blocked.

Table IV shows the results of an experiment on the reversal of blockage when the eggs were placed in the mercaptoethanol early after fertilization. The results of this experiment suggest that when the mercaptoethanol is applied during the earliest period of preparation for the first division, the effect is essentially a true block. The sum of the times from fertilization to the application of the chemical and from the removal of the chemical to cleavage is about the normal 50 minutes. There is no evidence that the eggs proceed very far toward division when exposed at these early times, nor that they

TABLE IV. *Recovery from blockage when mercaptoethanol was applied at early stages.*

Eggs were left in 0.1 *M* mercaptoethanol for 90 minutes, then returned to sea water.

Time after fertilization at which mercaptoethanol was applied (min.)	Percentage cleaved or cleaving at indicated times following removal of mercaptoethanol		
	25 min.	35 min.	45 min.
3	0	0	50
10	6	50	95
30	90+	95+	95+

retrogress. If retrogression is to be found, it will be found at the very end of the sensitive period, where, as has been mentioned, there was visual evidence of some disorganization. Reversal experiments at this stage were inconclusive because of our inability to establish cleavage times with a precision of a few minutes, and this point will be dealt with by morphological studies.

DISCUSSION

As was stated in the introduction, these experiments were designed to test a prediction concerning the participation of protein -SH groups in the formation of the mitotic apparatus. If there had been no effect of mercaptoethanol that could be related to the establishment of the mitotic apparatus—e.g., if the agent either killed the cells or permitted them to pass through division—the outcome would be considered negative and contrary to the prediction. The results that were actually obtained conform to the prediction, but do not thereby necessarily prove the hypothesis.

The observation that an agent blocking cell division is ineffective after the cell has passed a given point in its approach to division is, of course, a common one, applying to treatments of many kinds. No attempt will be made to review the literature fully. To cite a few recent reports, Bullough [3] in his comprehensive review concludes that agents affecting the energy supply of dividing cells are ineffective after metaphase, and Swann [13] describes in detail the sensitivity of sea urchin eggs to CO at various times before division. Butros [4] has reported that the cleavage of *Ilyanassa* eggs was not retarded by sodium azide, trichlorophenol, iodoacetic acid or chloral hydrate

if the eggs were exposed within 15–20 minutes of the time of division, although the same concentrations of these agents blocked division if applied earlier. Thus the gross fact that the sea urchin egg loses its sensitivity to mercaptoethanol at a given stage is not unique and does not distinguish between possible explanations of its effect.

Since these experiments were designed around a hypothesis that would assign to the SH of mercaptoethanol an immediate structural role rather than an indirect function by way of energy-supplying mechanisms, we should examine the results in these terms. According to the original scheme presented by Mazia [8], protein molecules are linked into chains by the formation of intermolecular S–S bridges, the polymerization involving the oxidation of protein SH. This would predict that conditions under which protein SH tended to be held in reduced form would prevent the establishment of the fibrous structure. Obviously the results are in conformity to the prediction to the extent that the growth of the mitotic apparatus is blocked by mercaptoethanol. In another communication, the details of the structure of the mitotic apparatus and of chromosome behavior under this treatment will be described.

The observed results do not require that the intermolecular bonding involving protein SH take the form of S–S bonding. Some recent results on the isolated mitotic apparatus, to be reported later, are consistent with the presence of alternative bonds involving SH, such as are implied in the observations of Bárány [1] on the polymerization of actin through SH groups, and of Madsen and Cori [7] on the association of subunits of phosphorylase. It is now clearer to us, as it was not earlier, that blockage of intermolecular bonding by an SH compound such as mercaptoethanol might merely reflect the effective competition of the added SH compound with the protein SH for the sites at which the intermolecular bridges would be formed. This possibility is presented as a reasonable alternative to the S–S bridge, which has not yet been disproved. Stern [14] has called the S–S bridging into question in relation to the interpretation of the role of glutathione. His conclusions may well be correct, but his experimental points are not referred to specified phases of spindle formation itself, and it is not clear whether they would expose fluctuations in protein SH that might occur in the brief period during which the mitotic apparatus is being built up.

There remains the question, why is it that mercaptoethanol will block the formation of the mitotic apparatus, but will not destroy it once it is formed? In another communication, we shall show that if it is applied just at the beginning of metaphase, it will actually destroy it in part. But it does not do so

after metaphase has begun, and we can only speculate on the reasons why. Perhaps the best basis for such a speculation is the now-classical conception of the "inaccessibility" of SH groups in certain configurations of proteins. This inaccessibility has been attributed by Benesch, Benesch and Rogers [2] to the involvement of the SH in hydrogen bonding. One can imagine that mercaptoethanol under neutral conditions might block the formation of such bonds but could not undo them once formed. Similarly, the reactivity of S–S groups in native proteins to reducing agents is variable [11].

It deserves repeating that the consistence of the data with the hypothesis that protein SH is involved in the intermolecular linkages by which the mitotic apparatus is formed does not constitute proof of such an hypothesis. An alternative interpretation of these data is that the mercaptoethanol is acting at a somewhat more remote level; for instance, as an inhibitor of some energy-yielding reaction. It must be admitted that the grosser aspects of the mercaptoethanol blockage are not very different from those of blockage by CO, dinitrophenol and other enzyme inhibitors. In finer details, there are some differences between the effects of mercaptoethanol, and of CO, for which the most complete data are available thanks to the work of Swann [13]. The latter does not block division fully in the sea urchin unless applied before prophase. If applied between prophase and metaphase it delays division, while between metaphase and telophase it has no effect on the time of onset of division but may inhibit its completion. The mercaptoethanol block may be applied right up to metaphase. It is either fully effective or ineffective. If the mercaptoethanol is applied at a time beyond the last point at which full blockage is possible, the cell divides at the same time as do the controls and divides completely.

It is not appropriate at this time to discuss in detail the question whether mercaptoethanol is acting directly on the assembly of protein molecules to form the mitotic apparatus, as is implied in the overall design of the experiments, or whether it too functions at the more remote level of inhibition of enzymes supplying the energy for mitosis. The relevant experiments, which will be reported at another time are: (1) Isolation of mitotic apparatus from blocked cells to observe whether mercaptoethanol merely arrests mitosis or whether it causes interpretable structural changes, and (2) tests of the possibility that mercaptoethanol is a metabolic inhibitor comparable to the more familiar reagents that block mitosis. The latter question has already been raised by Dustin [5], who speculates that the metaphase blockage of mouse bone marrow cells by *dimercaptopropanol* (BAL) is a consequence of enzyme inhibition due to metal-binding.

SUMMARY

1. Mercaptoethanol in adequate concentration (about 0.1 *M*) will block division of *Dendraster excentricus* eggs if applied at any time before metaphase. Since the time schedules of various echinoid eggs seem to be comparable, it may be useful to restate this finding in time units. In this case where the normal time from fertilization to first cleavage is 50 minutes, the mercaptoethanol block can be applied at any time from fertilization to about the fortieth minute.

2. If the mercaptoethanol is applied after metaphase, it has no detectable effect on the division that is about to take place, but will block the next division. The discontinuity between the sensitive period and the insensitive period is very sharp.

3. Concentrations of mercaptoethanol that are too low to block the first division may arrest cleavage at a subsequent division. In general, the lower the concentration, the more divisions will take place before blockage occurs.

4. The effect of mercaptoethanol is completely reversible.

5. Roughly, the time required for the blocked eggs to complete division when mercaptoethanol is removed is equivalent to the normal time required for the eggs to pass to division from the stage at which the mercaptoethanol was applied.

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PARTICLES EXHIBITING OXIDATIVE ENZYME ACTIVITY IN YEAST[1]

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Received August 29, 1957

During the synthesis of succinic dehydrogenase and of cytochrome oxidase induced by aeration of anaerobically grown yeast [9], a marked increase was observed in the refractile granules visible in the cells with the phase contrast microscope. These granules stain with Janus green B, with Nadi reagent, and with triphenyltetrazolium, and on this basis, have been described as mitochondria by various authors [15, 16, 8]. However, more recently other authors [3, 7] have proposed that other particles are the mitochondria in yeast, largely on the basis of differential Janus green B staining. In addition, Agar and Douglas [1] have identified mitochondria in electron micrographs of ultrathin sections of whole yeast cells on the basis of the presence of cristae, and have distinguished them from storage granules, whose contents are soluble in toluene. In view of the association of the oxidative enzymes with the particulate material of yeast [22] and of the association of oxidative enzymes with mitochondria generally, in all organisms, the particles exhibiting enzyme activity in cell-free preparations were examined with the electron microscope.

It was found that cytochrome oxidase activity is not associated with the refractile granules but rather with vesicle walls. The relation of these vesicle walls to structures visible in the whole cells with the phase contrast microscope and also to known properties of preparations of liver mitochondria is discussed. The variation in these structures with different growth conditions and particularly with the formation of the oxidative enzymes on aeration of anaerobically grown yeast is described.

[1] This work was supported in part by a grant from the Elsa U. Pardee Foundation and in part by Institutional Grants from the American Cancer Society, Inc., and the American Cancer Society, Southeastern Michigan Division.

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MATERIAL AND METHODS

Yeast strain LK2G12 and Fleischman commercial cake yeast were used in the studies of cell-free preparations. For these experiments, strain LK2G12 was grown aerobically in shake flasks in fat-supplemented medium, and the cells broken by shaking with glass beads and superbrite, as described previously [9], in 0.44 *M* sucrose, or in 0.05 *M* pH 6.8 phosphate buffer, or as otherwise described. The cell walls were removed by centrifuging at 1600 × *g* for 3 or 4 minutes and the cell-free supernatant centrifuged either in a Sorvall angle-head centrifuge or in an ultracentrifuge at 0°–4°C for the various speeds and times indicated.

The centrifuged fractions were assayed for cytochrome oxidase activity using the method described by Lucile Smith [23]. The protein content of the fractions was determined by the method of Lowry *et al.* [13].

A drop of appropriately diluted particles from each fraction was placed on a formvar-coated grid, fixed for 30 seconds with 1 per cent osmium tetroxide buffered at pH 7.4 with acetate-veronal buffer, allowed to dry, shadowed with chromium, and photographed with the electron microscope.

For the ultrathin sections of cell particles examined with the electron microscope, the cells were broken as above, the cell walls were removed, and the particulate material was sedimented by centrifuging for an hour at 197,000 × *g* in a Spinco analytical ultracentrifuge. The pellets so obtained were broken into 1 to 2 mm^3 pieces, which were fixed in pH 7.6 buffered osmium tetroxide and embedded in *n*-butyl methacrylate, following the procedure described by Palade [18]. The pellets were sectioned at $\frac{1}{40}$ to $\frac{2}{40}$ of a micron with a Porter-Blum ultramicrotome, using glass knives. These ultrathin sections were then mounted on formvar-coated specimen grids and examined.

To relate the structures observed in the electron micrographs of cell-free preparations with those visible in whole cells with the phase contrast microscope, the cell-free preparations were examined with the phase microscope. In addition, cells were broken under the microscope by pressure on the coverslip and the exudate examined immediately. The suspending media used were distilled water, 0.05 *M* pH 6.8 or 7.6 phosphate buffer, 20, 40 and 50 per cent bovine albumin (Fraction V, Armour and Co.) [2], and 0.22 *M*, 0.44 *M* and 0.88 *M* sucrose, with and without 7.5 per cent polyvinylpyrrolidone [17].

To study the variation in cellular structures during oxidative enzyme formation, strain LK2G12 was grown anaerobically both in fat-free medium, and in fat-supplemented medium [9], with and without shaking. The anaerobic yeast was then harvested; oxidative enzyme synthesis was induced by aerating the cells in a solution of 3 per cent glucose in *M*/15 KH_2PO_4, and the changes in cell structures observed with the phase contrast microscope.

This strain was also grown aerobically in S-3 medium and stained with Janus green B (certified for mitochondria) at various stages of the growth cycle according to the procedure described by Ephrussi *et al.* [7]. In addition, cells were stained with Janus green B using the procedure described by Bautz [3].

RESULTS

The relation between enzyme activity and the particles obtained in cell-free preparations

The cytochrome oxidase activity of various fractions of cell particles obtained from cells broken in phosphate buffer and centrifuged at different speeds, in a typical experiment, is given in Table I. It is apparent from the table that virtually all the activity is in the particles sedimentable at 26,000 × *g* or below. The amount of activity in each fraction varied somewhat with different preparations and with different yeasts, but virtually all of the activity always sedimented below 30,000 × *g*.

The nature of the particulate matter in the fractions assayed is shown in Fig. 1. The pellet obtained below 1600 × *g* (Fig. 1 *A*) contains cell walls, primarily, usually with less than 5 per cent of unbroken cells, as well as varying amounts of clumped particles, which probably account for the activity sometimes obtained in this fraction. The active fractions consist primarily of flattened vesicles, typically between 0.5 and 2 μ, in the fractions sedimented at 5000 × *g* (Fig. 1 *B*) and at 10,000 × *g* (Fig. 1 *C*) and between 0.1 and 0.5 μ in the material sedimented at 26,000 × *g* (Fig. 1 *D*). Occasional dense granules also sediment (Fig. 1 *C*, R.G.). Above this speed, a more or less homogeneous gelatinous material sediments (Fig. 1 *E*). The fatty layer appearing at the top of the centrifuge tube consists of fat droplets and of small dense spheres 0.3 to 0.5 μ in diameter similar to the refractile granules visible in the whole cells with the phase contrast microscope (Fig. 1 *F*).

TABLE I. *Cytochrome oxidase activity of centrifuged fractions of particulate material from yeast.*

Activity is expressed as k/min/mg protein. The cells were broken in 0.05 *M* pH 6.8 phosphate buffer by shaking with glass beads and superbrite [9]. Electron micrographs of the same fractions are shown in Fig. 1.

	Fraction		Activity		
	Av. × g	Time (min.)	Pellet	Supernatant	Fat
I	1,600	4	0	1.5	—
II	5,000	15	4.7	1.3	—
III	10,000	15	7.4	0.4	0.1
IV	26,000	15	7.1	0.1	0
V	197,000	60	0.4	0	0

The inactive 26,000 × *g* supernatant also contains very tiny dense particles about 30 mμ in size, that are shown most clearly in an unshadowed preparation of cells that were broken in 0.44 *M* sucrose and not centrifuged (Fig. 1 *G*). These particles appear the same as those attached to the thin filaments found in the ultrathin sections of the pellets sedimented at 197,000 × *g* (*P*, Figs. 2 and 3), and are of the same size order as the RNA-containing particles described by Palade in the endoplasmic reticulum of many kinds of cells [19], and also the small 24 mμ particles isolated from yeast by Chao and Schachman [4] and shown to carry out lipid synthesis by Klein [10].

It is apparent that cytochrome oxidase activity is associated with the vesicle walls sedimenting below 26,000 × *g*. The fatty layer, consisting of the refractile granules often previously described as yeast mitochondria, exhibits no enzyme activity. Such granules from cell-free preparations appear in small numbers in the pellet, but they are concentrated in the fatty layer. Variation in the fat content of the particles with different growth conditions can account for some granules sedimenting, and the fact that some are trapped by the descending vesicle walls, for others.

Ultrathin sections of the upper layer of the pellet obtained by centrifuging similarly prepared cell-particle preparations at 197,000 × *g* for an hour are shown in Fig. 2. Fig. 2 *A* shows typical vesicles between 0.5 and 1 μ, bounded by a smooth membrane and containing a filamentous, somewhat granular, matrix (*MC*). The vesicles resemble very closely the swollen rat liver mitochondria obtained by Watson and Siekevitz [24] when they sus-

Fig. 1.—Electron micrographs of the differentially centrifuged fractions of particles from yeast cells broken in 0.05 *M* pH 6.8 phosphate buffer and assayed for cytochrome oxidase activity. Scale marks indicate one micron.

A. Enzymatically inactive cell walls sedimented by centrifuging at 1600 × *g* for 4 minutes.

B. Enzymatically active vesicles sedimented by centrifuging the cell-free supernatant at 5000 × *g* for 15 minutes. The larger vesicles resemble the vesicles visible in the intact cell and in the cell exudate in the phase contrast microscope. The dark internal granules are the same size as the lipid granules, which are concentrated in the fatty layer, and which are sometimes observed inside or attached to the outside of vesicles in the cell exudate.

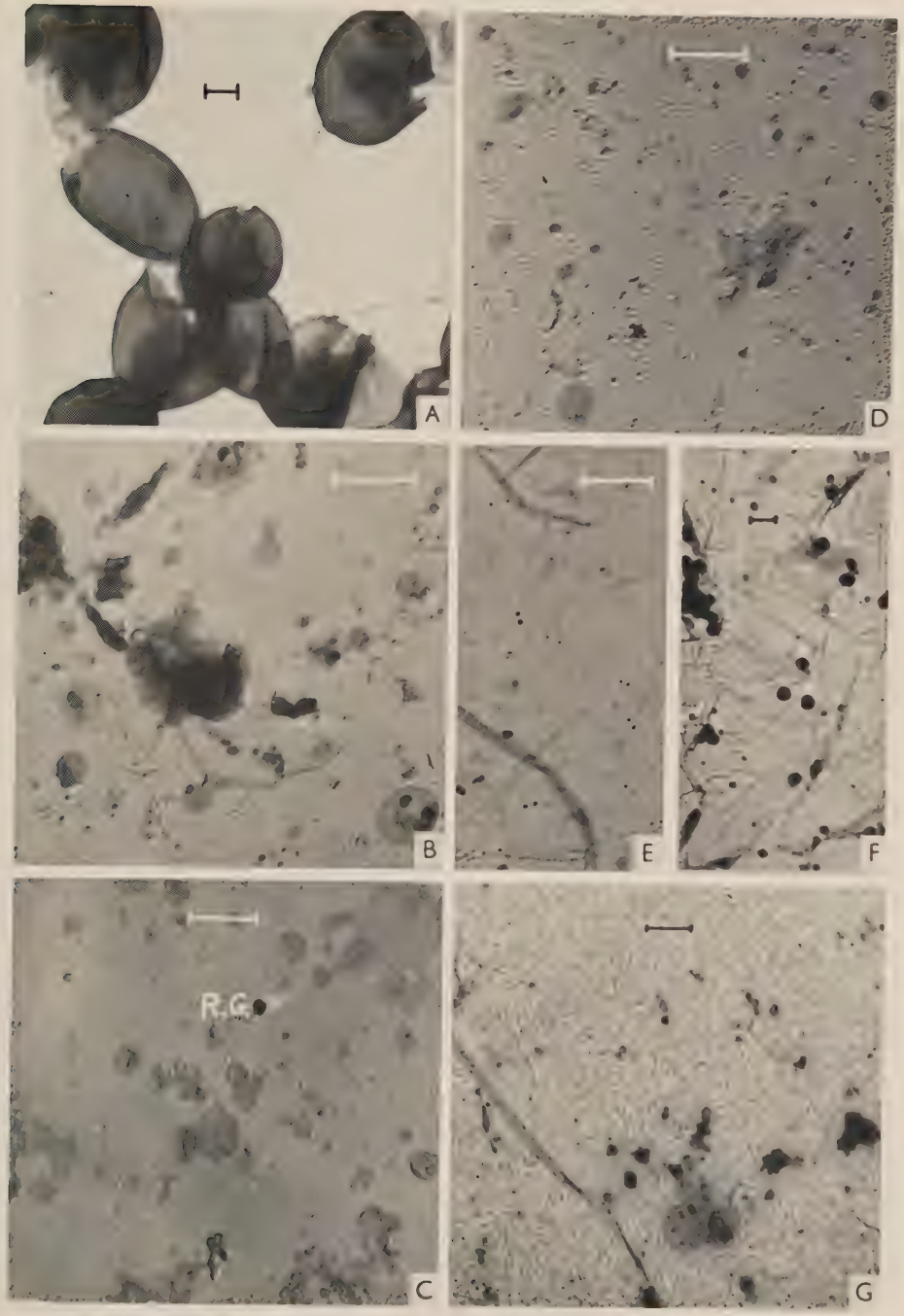
C. Enzymatically active vesicles sedimented at 10,000 × *g* for 15 minutes. One refractile granule (R.G.) is visible in this field.

D. Enzymatically active vesicular material sedimented by centrifuging at 26,000 × *g* for 15 minutes.

E. Enzymatically inactive amorphous material sedimented by centrifuging at 197,000 × *g* for an hour.

F. Enzymatically inactive fatty layer consisting largely of dense granules resembling the lipid granules visible in the intact cells with the phase contrast microscope.

G. Three vesicle walls and many approximately 30 mμ particles from cells broken in 0.44 *M* sucrose and not centrifuged. The preparation is not shadowed.


A
D
B
E
F
R.G.
C
G

pended rat liver mitochondria in water. Interspersed between the swollen mitochondrial vesicles are spongy membranes (*M*) enclosing some of the very fine strands studded with the approximately 30 mμ particles (*P*) that are also scattered throughout the preparation. These latter two constituents may be derived from disrupted mitochondria, but the particles attached to the strands appear larger, occur more frequently and appear more dense than those in the mitochondrial matrix. The fine strands of particles closely resemble remnants of the endoplasmic reticulum found by Watson and Siekevitz [24] as contaminants in their preparations of disrupted liver mitochondria. Furthermore, the spongy matrix of membranes resembles the highly vesiculated cytoplasmic exudate observed in the phase contrast microscope when cells are broken in water, buffer, or sucrose solutions.

Fig. 2*B* is a lower magnification of a different area of the same section as Fig. 2*A*, showing the types of structures found in the pellet, including one vesicle enclosing several smaller internal vesicles (*MC'*). This latter vesicle also resembles some of the partially disrupted liver mitochondria obtained by Watson and Siekevitz [24].

Visible in the ultrathin section in Fig. 3*A* are: (1) similar vesicles (*MC*) around 0.5 μ in diameter with a fairly homogeneous internal matrix; (2) round dense granules up to 0.2–0.3 μ in diameter (R.G.), which resemble the refractile granules visible in the whole cells with the phase contrast microscope, and which are concentrated in the upper fatty layer after centrifuging; (3) bits of membrane; (4) vesicles of various sizes containing the particle studded strands (*P*).

One unusually large vesicle (*V*) clearly shows the internal granular filaments, two dense round highly osmiophilic structures about 0.2–0.3 μ in size, and several smaller flat round bodies. This large vesicle resembles the vesicles observed in the whole cells and in the cell exudate with the phase contrast microscope (Fig. 3). The refractile granules were always observed outside the vesicles in the intact cell, but occasionally one or two were seen attached to the vesicles or moving about inside the vesicles found in the cell-

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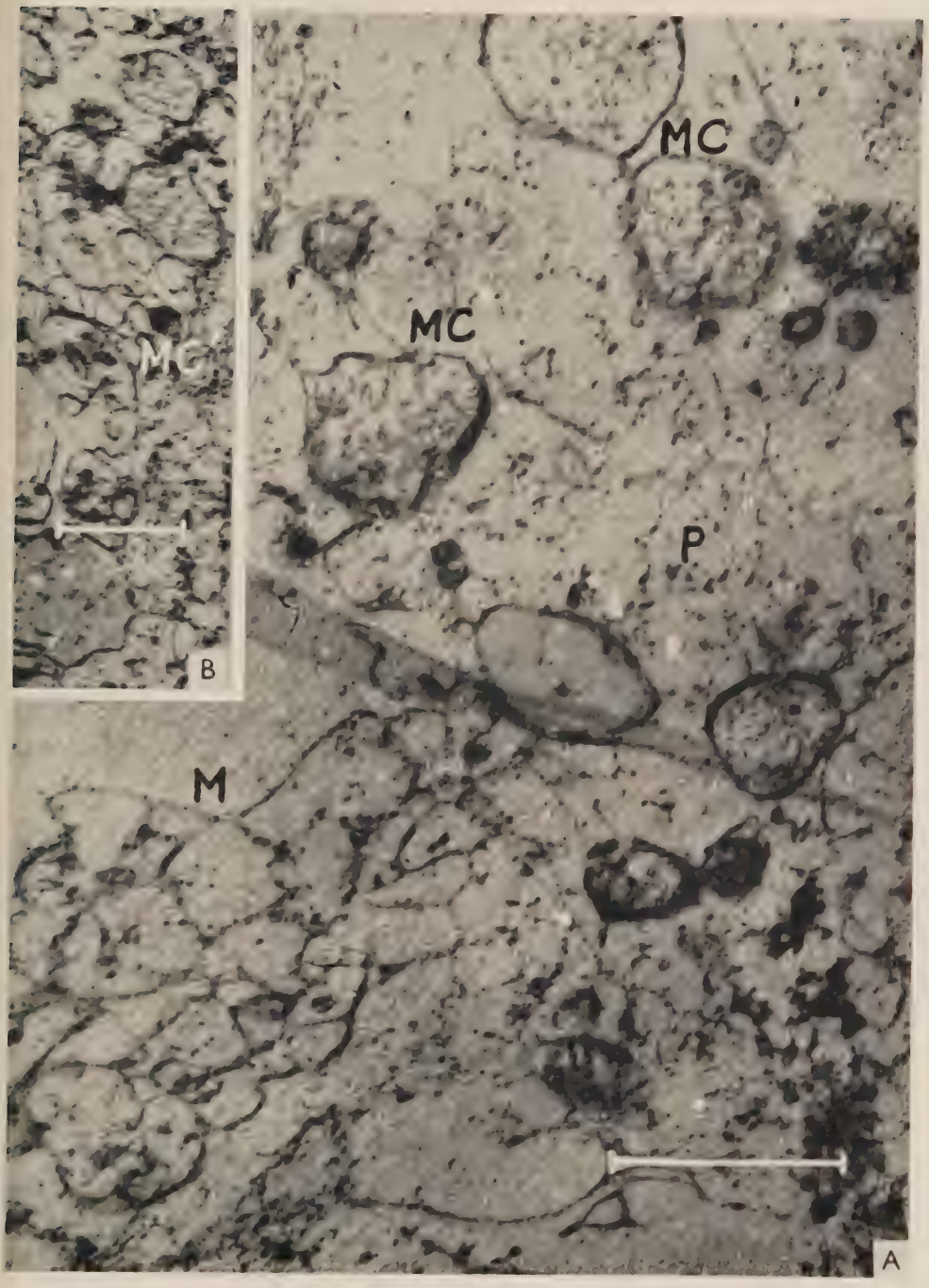
Fig. 2.—Ultrathin sections of structures in the centrifugate of a cell-free preparation of yeast. Scale marks indicate one micron.

A. Ultrathin section through the upper layer of the pellet of particles from yeast cells broken in 0.05 *M* pH 7.6 phosphate buffer and centrifuged at 197,000 $\times$ *g*, after removal of the cell walls. This section has been enlarged in order to compare the very fine strands of particles (*P*) found throughout the pellet with the faintly granular filamentous matrix filling the smooth walled vesicles of 0.5 to 1 μ (*MC*). Spongy membranes (*M*) are also visible in this section.

B. A lower magnification of an area of the same ultrathin section showing a vesicle filled with smaller internal vesicles (*MC*).


MC
MC
P
M
A
MC
B

free exudate. Lipoidal "dancing bodies" often appeared in the central vacuole following pressure on the cell (Fig. 3*F* and *G*) but these were a micron or more in diameter and gave no evidence of having a structure, since the droplets coalesced on contact.

The vesicles in sectioned pellets of cellular debris of cells broken in 0.44 *M* sucrose and in 40 per cent albumin were similar in appearance to those broken in water or in buffer. In contrast to liver mitochondria, 0.44 *M* sucrose did not prevent swelling of the mitochondria. In whole cells exposed to this concentration of sucrose the cytoplasm rapidly vesiculated even prior to breakage. The approximately 30 mμ particles readily separated from the strands to which they are attached, in sucrose (Fig. 1 *G*) as well as in the other suspending media. The mitochondrial vesicles in 40 per cent albumin, however, were somewhat less swollen; the larger structures being typically 0.2–0.3 μ × 0.5–0.7 μ in size and in many of them there was some evidence of a striated internal structure. The use of albumin preparations to prevent cytoplasmic swelling is being studied further.

Effect of growth conditions on cell structures

The structures visible in typical aerobic cells with the phase contrast microscope and shown in Fig. 3*B* are as follows.

Fig. 3.—Relation between structures seen in electron micrographs of cell-free preparations and those visible in whole cells and in crushed cells, with the phase contrast microscope.

A. Cross section of the upper layer of the pellet of centrifuged cell particles from cells broken in 0.05 *M* pH 7.6 phosphate buffer. There is a large vesicle (*V*) enclosing two dense bodies about 0.3 μ in diameter (R.G.) and several smaller round structures as well as fine strands of tiny particles (*P*), such as are scattered throughout the pellet. The smaller more uniform vesicular structures (*MC*) with a homogeneous appearing interior resemble swollen mitochondria. Scale mark indicates one micron.

B. Aerobic cells showing the large empty looking central vacuole, the small dark refractile granules and the region displacing the vacuole in some cells which has been described as the nucleus.

C. Large cell grown on Lindegren's presporulation medium showing the grey areas sometimes observed in the cytoplasm.

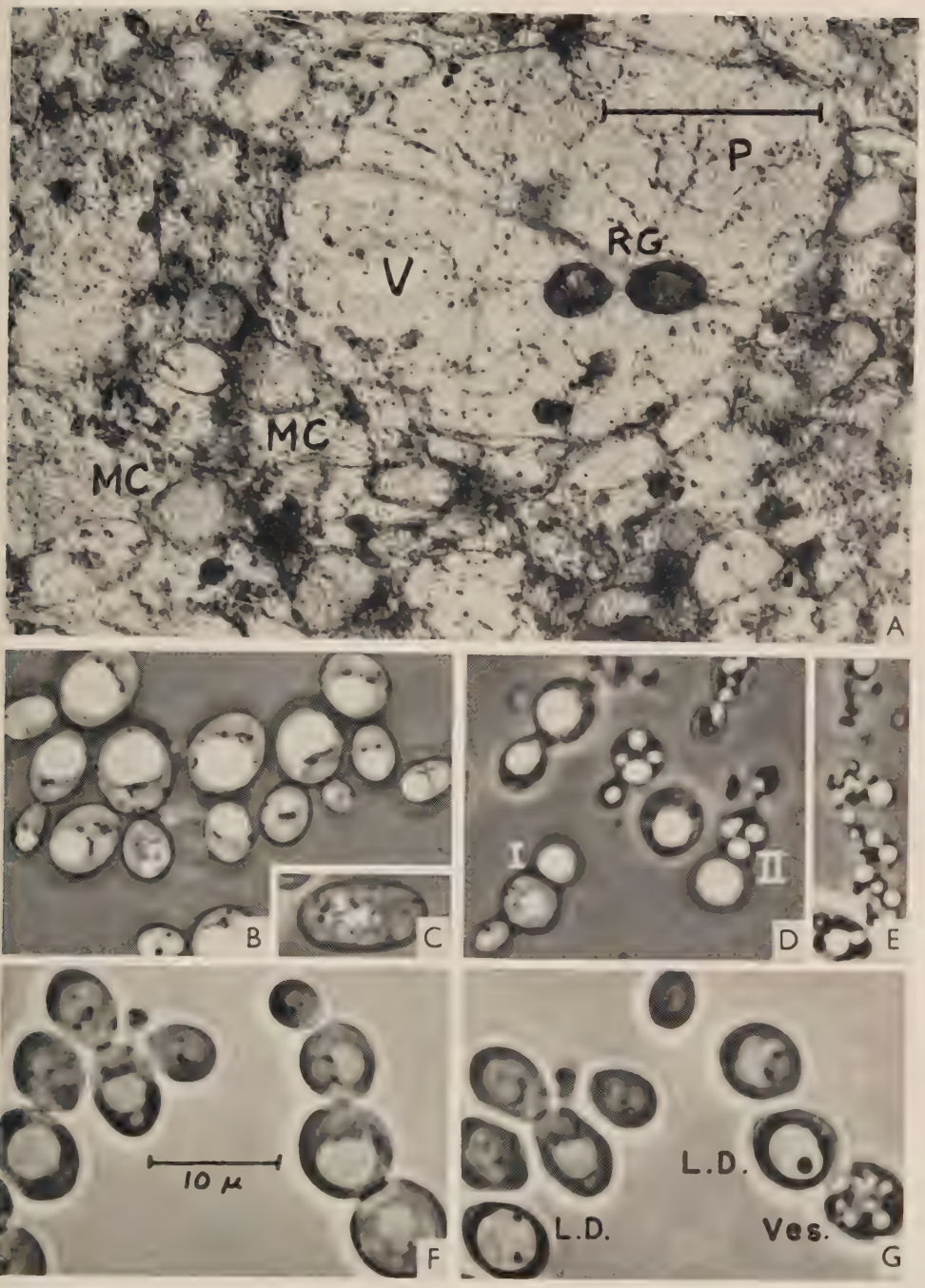
D. Cells suspended in 40 per cent albumin that have been crushed slightly. At I a drop of cell sap is beginning to flow out of the cell; at II sufficient cell sap has flowed out to produce a striking contrast between the vesicles and the surrounding cytoplasmic matrix remaining inside the cell.

E. Cell exudate showing intact vesicles in albumin.

F. Aerobic cells showing large central vacuoles in some of the cells and faint outlines of multiple vesicles in others, as well as refractile granules.

G. The same field of cells as in Fig. 3 *D* after slight pressure on the coverslip. Note the appearance of lipid droplets (L.D.) in two of the large vacuoles, and also the marked vesiculation of one cell (Ves.).

In *F* and *G* the cells are suspended in water and photographed with a Spencer B minus M phase contrast objective; in all the other figures, the cells are suspended in 40 per cent albumin and photographed with a dark M phase contrast objective.


P
RG
V
MC
MC
A
B
C
I
II
D
E
10 μ
F
L.D.
L.D.
Ves.
G

(1) There is a large central vacuole which usually appears empty, but often contains a rapidly moving lipid droplet after the cell has been subjected to slight pressure (Fig. 3*F* and *G*).

(2) There are also numerous smaller vesicles, which vary in size and in density with growth conditions. These are often barely distinguishable from the surrounding cytoplasm, but become more distinct with slight pressure on the cells (Fig. 3*D*, *F*, and *G*). When cells are suspended in 40 per cent albumin, and mashed slightly, as the cell sap flows out, the cytoplasmic matrix inside the cell becomes darker due to the higher refractive index of the residual material, and the vesicles become very bright by contrast (Fig. 3*D*). The vesicles remain intact when the cells are broken in a 40 per cent albumin solution (Fig. 3*E*) but expand in the other suspending media thus demonstrating the semipermeable nature of their membrane. In addition, when cells are broken in water, buffer, or sucrose solutions, as the cytoplasm flows out of the cell, it immediately bursts into a string of vesicles, thus suggesting a submicroscopic vesicular organization of the cytoplasm.

(3) The refractile granules (Fig. 3 *B* and *F*) are visible throughout the cytoplasm, often outlining vesicles, and frequently exhibiting Brownian movement. They appear in the exudate unchanged as to number and appearance, regardless of the suspending medium.

Fig. 4.—Changes in cellular structures during aeration of anaerobically grown yeast cells in glucose–buffer solution.

A. Freshly harvested anaerobic yeast grown to the stationary phase standing in fat-free medium showing the reduced number of refractile granules and vesicles.

B. The same cells after 15 minutes aeration in glucose–buffer solution showing the very rapid appearance of fine granules. Two dead cells, with bright halos, are present in the field.

C. The same cells after 60 minutes aeration, showing further increase in size and number of refractile granules.

D. The same cells after 4 hours aeration, showing the further increase in size and number of granules as well as marked vesiculation of the cytoplasm.

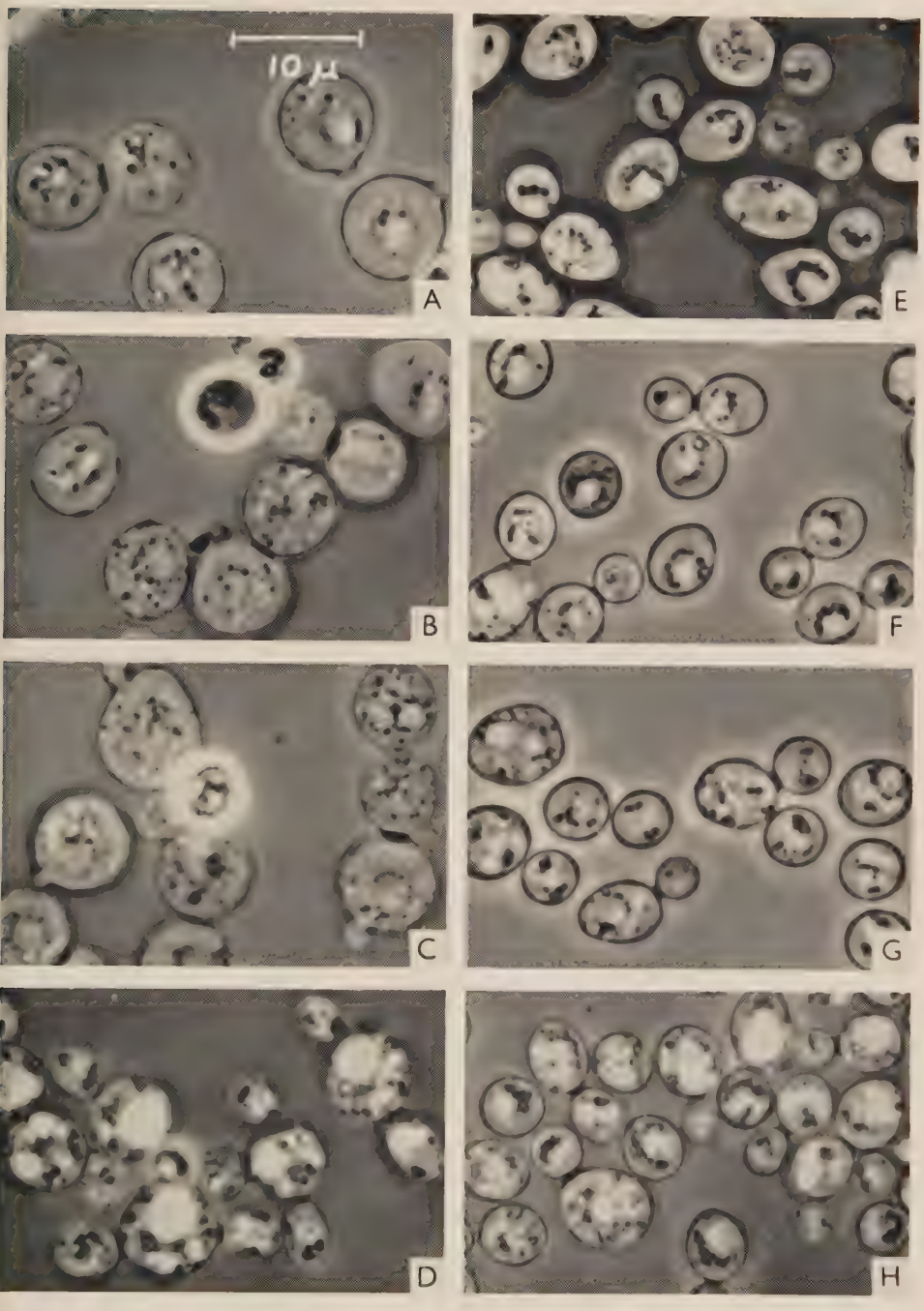
E. Freshly harvested anaerobic cells grown to completion of growth with shaking in fat-supplemented medium, showing the large number of granules and also the presence of vacuoles in the cells with the granules typically outlining the vacuoles.

F. The same cells after 15 minutes aeration in glucose–buffer solution showing little if any change in the cellular structures.

G. The same cells after 60 minutes aeration showing the appearance of many small vesicles and relatively little change in the granules.

H. The same cells after 3 hours' aeration showing the presence of large numbers of small vesicles and granules.

All photographs are the same magnification; the size of the cells is indicated by the 10 μ mark in *A*. The cells are suspended in 40 per cent albumin and photographed with the dark *M* objective of a Spencer phase contrast microscope.


10 μ
A
E
B
F
C
G
D
H

(4) Occasionally, as in cells grown in Lindegren's presporulation medium [11], small round or irregular oblong areas are visible which appear as slightly darker grey than the surrounding cytoplasm (Fig. 3*C*). These areas are of the same size and position in the cell as the mitochondria shown in ultrathin sections of yeast cells grown on presporulation medium by Agar and Douglas [1].

(5) The vacuole may be distorted in the region described by various authors [1, 6] as the nucleus, by a structure (Fig. 3*B*, *F* and *G*) which is not generally visible in the phase contrast microscope except as it is outlined by refractile granules.

The increase in granules and in vesicles with aeration of the anaerobic cells grown standing in fat-free medium is shown in Fig. 4*A* to *D* and with aeration of the anaerobic cells grown on fat-supplemented medium with shaking in Fig. 4*E* to *H*. These growth conditions correspond respectively to those giving the lowest and the highest yields, and the slowest and the most rapid rates of oxidative-enzyme formation during aeration in gluose-buffer solution.

In addition to the lower number of refractile granules in anaerobically grown yeast, especially that grown in the fat-free medium, there is also a twofold or more decrease in the number of granules during rapid logarithmic growth as compared with cells in the later stationary growth phase regardless of the method of growth.

Comparison of the cells of the anaerobic harvest (Fig. 4*A* and *E*) shows that the cells grown in fat-free medium differ from those grown on the supplemented medium in the following ways: (1) they have fewer and often larger refractile granules, (2) the cells are larger, and (3) the cytoplasm lacks the organization of the cells grown on the fat-supplemented medium; i.e. vacuoles and vesicles are not as apparent and the refractile granules are not clumped around the vesicles. In addition, there are more dead cells among the cells grown on the fat-free medium, and the cells are more fragile, vesiculating readily with slight pressure.

Fig. 4*B* shows the very rapid formation of a very large number of tiny refractile granules that occurs within 15 minutes of aeration of the fat-free anaerobically grown cells.

Two changes occur in the gross appearance of the centrifuged cell-free preparation corresponding to these changes in cell particles during enzyme formation: (1) The turbid fatty layer of refractile granules increases in amount, and (2) the upper fluffy layer of the pellet consisting of the larger vesicle walls increases several fold. These increases were most marked in

the slowly adapting anaerobic yeast grown on fat-free medium, since the initial levels were very low.

It was not possible to correlate the structures seen in unstained cells with the phase contrast microscope with those described as mitochondria following staining with Janus green B by Bautz [3], and also by Ephrussi *et al.* [7], because of the marked coagulation of the cytoplasm and consequent obscuring of structures and disappearance of the vacuoles and vesicles visible in the unstained cells.

DISCUSSION

Cytochrome oxidase activity in the cell-free preparation of yeast is associated with vesicle walls varying from 0.2 μ to over 1.0 μ. Recently Agar and Douglas [1] have shown typical mitochondria with cristae mitochondriales in ultrathin sections of whole yeast cells. Watson and Siekevitz [24] have shown that liver mitochondria in cell-free preparations swell to become smooth walled vesicles, of a micron or less in diameter, filled with a fine filamentous matrix and occasional granules. They have also shown that with more drastic treatment, as with sodium deoxycholate, the mitochondria disrupt completely to form membranes and very small empty vesicles or small vesicles within larger vesicles, and further [21] that succinoxidase and cytochrome oxidase activities are associated with these membranes.

The present studies show that the mitochondria in cell-free preparations from yeast are very similar to those of liver (1) in the swelling of the mitochondria in hypotonic solutions, (2) in the appearance of the swollen mitochondria, and (3) in the association of cytochrome oxidase activity with the disrupted mitochondria.

However, we have not been able to prevent swelling of mitochondria nor the vesiculation of the cytoplasm in cell-free preparations using the suspending media usually employed to preserve liver mitochondria, such as 0.44 *M* and 0.88 *M* sucrose, with and without 7.5 per cent polyvinylpyrrolidone [17]. The better preservation of cell contents obtained with 40 per cent albumin suggests that the higher tonicity in yeast cells [2] as compared with animal cells can account for the ineffectiveness of the sucrose solutions. However, the vesiculation of the cell contents prior to breakage, regardless of the breakage method employed, including the Nossal disintegrator [12], suggests that the tough cell wall of yeast may result in some injury to the cell contents prior to breakage.

Thus, the possible origins of the vesicles found in the exudate of yeast

cells broken in media such as are usually used in the preparation of cell particles for enzyme assay are (1) the mitochondria, (2) the vesicles and vacuoles visible in the intact cell, and (3) smaller vesicles not visible in whole cells with a light microscope, such as the cisternae common to the endoplasmic reticulum of cells, in general.

Klein [10] has reported that fat synthesis in cell-free preparations of yeast is associated with the small particles (20–30 mμ in diameter) which sediment at 100,000 $\times$ g. On the other hand Corwin *et al.* [5] have found virtually all of the lipogenic activity of cell-free preparations of yeast in the particles sedimenting at 11,500 $\times$ g. Such 20–30 mμ particles in our preparations have been found both free (Fig. 1*G*) and attached to strands resembling elements of the endoplasmic reticulum (Fig. 2 and 3*A*). It appears from this that the endoplasmic reticulum may be the source of these granules. Thus, the less drastic breaking methods employed by Corwin can account for his obtaining lipogenic activity in larger cellular elements.

The nature and function of the larger refractile lipid granules is not known, although Bautz has suggested that they are comparable to the spherosomes of higher plants [3], and they are similar in size and appearance to the lipid granules of animal cells [20]. The correlation found between the anaerobic growth conditions allowing for (1) high yield, (2) increase in number of lipid granules, (3) increase in membranous structures in the yeast cytoplasm, and (4) the ability to synthesize oxidative enzymes on exposure to air, suggests a relation between the structure of the anaerobic cell and its oxidative enzyme synthesizing capacity [9]. This is not unexpected in view of the fact that the oxidative enzymes being studied are bound to structural elements of the cell and also have been shown to contain lipid [14].

The cell-free preparations were assayed only for cytochrome oxidase, because of the greater stability and ease of preparation of this enzyme. However, previous assays of the succinic dehydrogenase complex have shown that both it and cytochrome oxidase increase similarly during adaptation, and that it is attached to the particulate matter similarly sedimented by centrifugation [9].

SUMMARY

1. Cytochrome oxidase activity in a cell-free preparation from yeast is associated with flattened spherical vesicles ranging from 0.2 μ to over 1 μ and sedimenting between 5000 $\times$ g and 26,000 $\times$ g.

2. The 26,000 $\times$ g supernatant contains minute particles approximately 30 mμ in diameter, and the fatty layer at the top of the centrifuge tube con-

tains fat droplets and lipid granules about 0.3 μ in diameter. Neither of these particles exhibit cytochrome oxidase activity.

3. The structures found in ultrathin sections of the particle preparation are compared with those observed in whole yeast cells and in the exudate of broken cells with the phase contrast microscope. They are also compared with the known structures of cell-free preparations from liver. The enzymatically inactive lipid granules found in the fatty layer are identical with the refractile granules visible in the whole cell which have often been described as mitochondria on the basis of their staining reactions. Some of the vesicles appear to be derived from swollen mitochondria and others from other cytoplasmic elements.

4. Variation in these cellular structures with growth conditions has been studied, particularly the reduction in lipid granules in yeast grown under anaerobic conditions, and the marked increase in these granules with aeration of such anaerobic cells.

The authors would like to thank Dr. G. E. Palade for his very helpful discussion and suggestions relating to the preparation and ultrathin sectioning of the cell-free particulate material.

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SITES OF NUCLEIC ACID SYNTHESIS IN THE MOUSE VISUALIZED BY RADIOAUTOGRAPHY AFTER ADMINISTRATION OF C^{14}-LABELLED ADENINE AND THYMIDINE

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Received August 31, 1957

Since adenine is utilized by animal tissues for the synthesis of both ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) [7, 11, 21, 29], radioautographs obtained after injection of C^{14}-labelled adenine may be used to trace both nucleic acids in sections. If RNA is extracted by either ribonuclease [24, 25], hydrochloric acid [14] or perchloric acid [24], the radioautographic reactions which are then observed are attributed to newly formed DNA only [14, 24, 25]. Furthermore, a radioautographic comparison of extracted and non-extracted sections may indicate the location of newly formed RNA.

In the past, radioautographic studies with labelled adenine have been limited to bone marrow cells *in vitro* [14] and testis *in vivo* [24, 25, 28], with a few other tissues receiving brief mention [25]. The purpose of the present investigation is to examine the incorporation of labelled adenine into the nucleic acids of a number of tissues using ribonuclease-treated and control sections.

A small amount of C^{14}-labelled thymidine became available while the investigation was under way. Since this nucleoside is taken up in significant amounts only into DNA [8, 10, 26], the whole sample was used in one animal in the hope of confirming the distribution of newly formed DNA.

MATERIALS AND METHODS

Groups of male mice were given a single subcutaneous injection of adenine-8-C^{14} with an activity of 100 μc per 17 mg (Radiochemical Centre, Amersham, England). A first group consisted of three 12-g C_3H male mice injected subcutaneously with 0.5 ml of a saline solution containing 5.5 mg adenine per ml (16 μc) and killed 8, 24 and 72 hours later. The second group was used in a repeat experiment and included three 15-g C_3H male mice, which were given approximately 0.42 ml of saline contaning 8.9 mg adenine per ml (22 μc) and again killed at 8, 24 and 72 hours. Finally, in a

subsidiary experiment, two recently born hybrid mice weighing 1.4 g received 0.05 ml of saline containing 5.5 mg adenine per ml (1.6 μc) and were sacrificed 8 hours later.

Over 30 organs and tissues were removed, fixed in Carnoy and sectioned at 6 μ. Some sections were incubated in a solution of 1 mg of ribonuclease (Worthington) per ml distilled water at 37°C for 2 hours, while controls were similarly incubated in distilled water [23]. Staining of the enzyme-treated and control sections of liver, pancreas, cerebrum and other tissues with a polychrome toluidine blue solution [2] confirmed that the basophilic material of the cytoplasm, which has been shown to be composed of RNA [33], is more or less completely removed by the enzyme treatment. Unstained and hematoxylin-eosin-stained slides of both enzyme-treated and control sections were processed for radioautography by coating with NTB3 liquid emulsion [13]. Exposure times ranged from two months to one year.

A few slides were immersed in 10 per cent trichloracetic acid at 90°C for 30 minutes for removal of both RNA and DNA [22] and were then radioautographed.

Finally, a sample of 489 μg of thymidine-2-C^{14} with a specific activity of 3 μc/micromole (about 6 μc altogether) was injected into a 12-g C_3H male mouse. The animal was sacrificed 24 hours later and radioautographs were made as above.

RESULTS

After injection of adenine-C^{14}, the radioautographs of control sections which had not been subjected to ribonuclease varied considerably in intensity from tissue to tissue (Fig. 1), but in every instance the reactivity was reduced by treatment of the sections with ribonuclease, as may be seen by comparing corresponding tissues in Figs. 1 and 2. Furthermore, the reactions were completely removed by treatment with hot trichloracetic acid.

In the first section of the results, the distribution of the reactions persisting after ribonuclease treatment will be described in the 12 15 g mice given adenine-C^{14}. In the second section, an attempt will be made to assess the location and intensity of whatever radioactive material was removed by ribonuclease in these animals. The third section will deal with two subsidiary experiments, namely, the radioautographic study of adenine-C^{14} in newborn mice and of thymidine-C^{14} in a 12-g mouse.

Ribonuclease-stable Radioactivity

Examination under low power of unstained sections from animals sacrificed 8 hours after adenine-C^{14} injection (Fig. 2, top) showed a considerable difference in the radioautographic reactions of various tissues: intense in spleen (*Sp*), small intestine (*I*) and colon (*C*), weak in testis (*Te*) with little or no reaction in a series of other organs and tissues, such as pancreas (*P*), muscle and bladder. With time, most reactions decreased in intensity as

may be seen by examining the organs just mentioned at 24 and particularly 72 hours after injection (middle and bottom of Fig. 2 respectively).

Under high power it was apparent that, except for a light, diffuse background, the reactions consisted of tiny individual foci, which were clearly distinguishable whenever exposure was adequate (Figs. 9, 14 and 19). When the small foci were well separated from one another, as in connective tissue (Fig. 25), it was noted that the reactions were located over nuclei. In the intestine the band-like reactions produced by the epithelium (Figs. 4 and 5) overlay the basal rows of nuclei but not the apical portion of the cells (as may be seen above the arrow in Fig. 5).

An exception to the rule that reactions are attributable to nuclei was the diffuse background over many organs, particularly pronounced in the case of liver (Fig. 2, *L*). Moreover, in the kidney of all 12–15 g mice, a few dense blotches were scattered over both cortex and medulla, without it being possible to assign them to definite structures.

Of the typical reactions, the most pronounced ones were observed in gastrointestinal and certain other epithelia, in hemopoietic organs and in connective tissue.

Gastro-intestinal epithelia. –A definite radioautographic reaction was seen over the linings of stomach (pyloric and body region), small and large intestine. Only the reactions in *jejunum* will be described in some detail. At 8 hours (Fig. 4), the epithelium of the crypts was overlaid by an intense blackening, but that of the villi was not. (A focus of reaction was occasionally seen over connective tissue cells in the core of the villi.) A large percentage of the nuclei of chief and goblet cells, but not those of Paneth cells, were overlaid by reactions. Moreover, since advanced prophases and metaphases border

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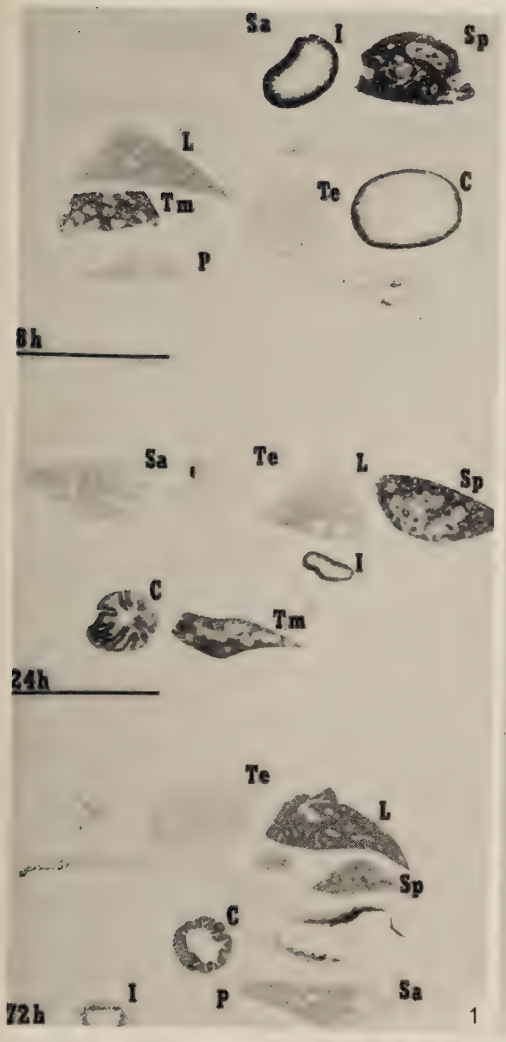
Unstained radioautographs of organs from 12-g mice injected with C^{14}-labelled precursors of nucleic acid. × 4.5.

Fig. 1.—From above downwards, radioautographs at 8, 24, and 72 hours after injection of adenine-C^{14}. The reactions (which may be attributed to both newly formed RNA and DNA) are, at 8 and 24 hours, intense in spleen (*Sp*), small intestine (*I*), colon (*C*), and thymus (*Tm*), moderate in liver (*L*), and weak in testis (*Te*), submaxillary (*Sa*) and pancreas (*P*). By 72 hours the reactions are decreased in intensity in spleen, small intestine and colon.

Fig. 2.—Same as Fig. 1, except that the sections were treated with ribonuclease. The reactions (which may be attributed to newly formed DNA only) are much less intense than in Fig. 1, but the pattern shows several similarities. (The difference in intensity between Figs. 1 and 2 was actually greater than shown, since the photograph illustrated in Fig. 2 had to be overexposed to bring out detail.)

Fig. 3.—Radioautographs at 24 hours after injection of thymidine-C^{14}. The distribution of radioactivity is similar to that observed after adenine-C^{14} injection and ribonuclease treatment (Fig. 2), except for the lack of reactivity in liver here.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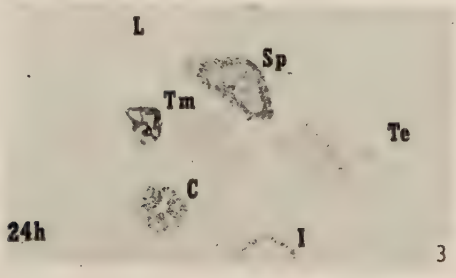
L
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the lumen of the crypts, they may be readily distinguished; and it appeared that some, but not all, were covered by silver granules.

At 24 hours (Fig. 5), some reaction seemed to extend to all crypt nuclei, except those of Paneth cells, but the most intense response of the emulsion was seen over the lower third or half of the villus epithelium (above the arrow in Fig. 5). By 72 hours (Fig. 6), most of the reaction was distributed over the upper two thirds of the villi, with a decreasing gradient from the tips down to the crypts. Furthermore, the intensity of the reaction over the villus tip region was less than that observed over the villus base at the 24-hour interval (compare Figs. 5 and 6).

The radioautographic pattern for *ileum* (8 hours, Fig 18) and *duodenum* (24 hours, Fig. 17) was the same as for jejunum, except that in duodenum a reaction was already present over the tips of at least some villi at 24 hours (Fig. 17), while only traces of reaction were left by 72 hours. In the *descending colon* the reaction was initially over some of the epithelial nuclei in the lower third of each crypt (Fig. 14) and subsequently became distributed throughout with a maximum over the cells bordering the gut lumen (Fig. 15, arrows). In the *ascending colon* at 8 hours the response of the emulsion predominated in an area half-way up the crypts (Fig. 12), but the 72-hour pattern again showed a maximum near the gut lumen (Fig. 13). At that time a reaction was occasionally found over epithelial cells that were free in the lumen.

The *stomach* at 8 hours had a band of reaction somewhat above the middle of the mucosa in the body region (Fig. 16) and somewhat below the middle in the pyloric region. At 72 hours, the reaction predominated at the surface in both regions.

Other types of epithelia.—The *stratified squamous* epithelium of oesophagus,

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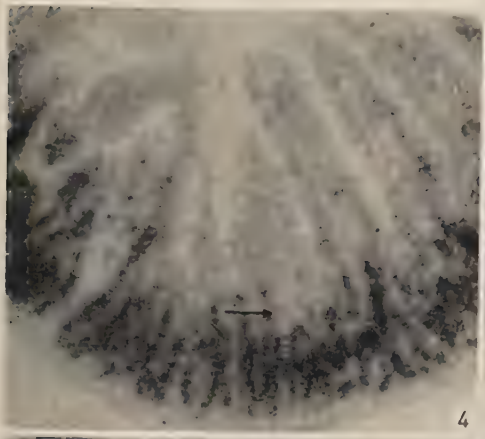
Radioautographs of RNAse-treated tissues from 12-g mice given an injection of adenine-C^{14}.

Figs. 4, 5, 6.—H & E stained radioautographs of jejunum at 8, 24, and 72 hours after injection. 2-month exposure. The junction of crypts and villi is shown by arrows. Against a weakly stained background, the reactions observed at 8 hours (*Fig. 4*) are arranged in parallel bands corresponding to the nuclei of the crypt epithelium. At 24 hours (*Fig. 5*), they extend part way up the sides of the villi and by 72 hours (*Fig. 6*), they line the sides and tips of the villi. × 107.

Figs. 7, 8, 9.—Unstained radioautographs of spleen at 8, 24, and 72 hours after injection. 6-month exposure (same tissues as appear in Fig. 2). In the 8-hour illustration (*Fig. 7*), the light area extending across the center of the picture corresponds to the white pulp, while the dark areas at top and bottom are due to the red pulp. The white pulp displays four dark masses which are due to the intense reactions over the germinal centers of lymphatic nodules. The rest of the white pulp, which is made up of the cortical portions of the nodules, shows scattered foci corresponding to individual cells. The red pulp contains cords of hemopoietic tissue which produce intense reactions alternating with unreactive areas. At 24 hours (*Fig. 8*), the pattern of reaction is similar, but the intensity appears to be decreased. By 72 hours (*Fig. 9*), only scattered small foci corresponding to individual cells persist in the white as well as in the red pulp. × 53.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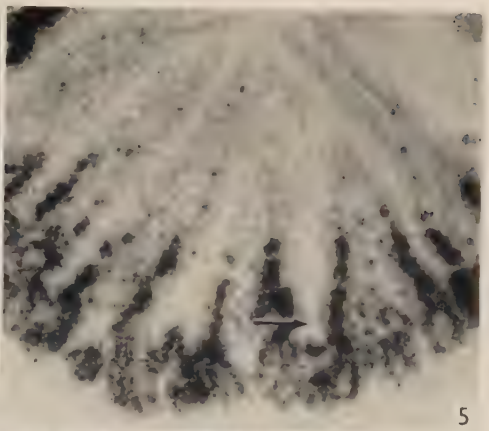
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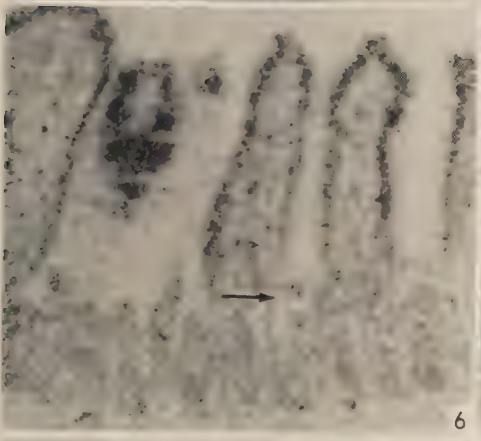
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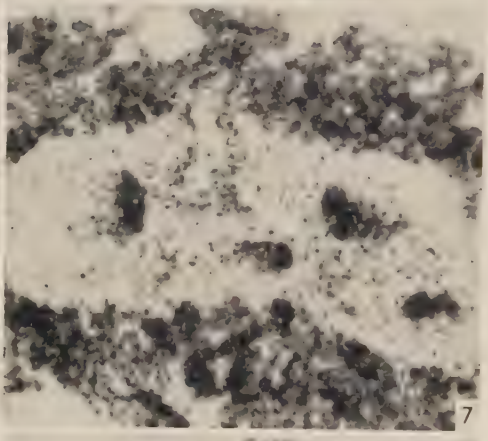
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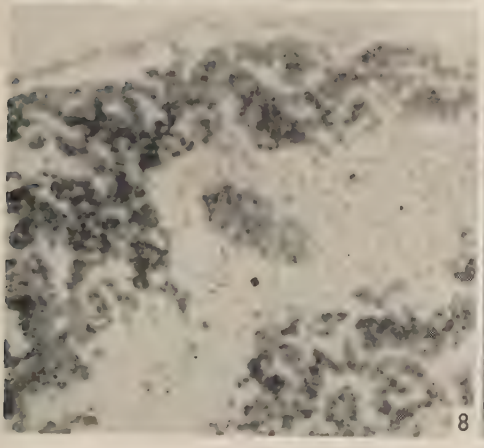
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gums, tongue, and plantar epidermis at 8 hours displayed scattered foci of reaction over the basal layer. In the oesophagus, such foci seemed to increase in number but remained near the basement membrane at 24 and 72 hours. This was true also of most of the gum epithelium, except in the regions in immediate contact with the teeth, where an intense reaction was observed at 8 hours over the basal layer, while at 72 hours the reaction foci were scattered at various levels within the epithelium from the basal layer to the surface. In the tongue at 8 and 24 hours, weak and strong reactions alternated over the basal layer; the weak ones corresponded to the interpapillary spaces (Fig. 10, arrow) and the strong ones to the papillae themselves. This differentiation was also discernible at the 72-hour period (Fig. 11), when the malpighian layer was radioactive to a lesser extent in the areas between papillae (arrow) than within the papillae themselves. The thin epidermis from the skin of the lateral abdominal wall showed widely scattered foci, which may increase in number with time. The malpighian layer at the base of hair follicles produced an intense reaction (Fig. 21), while a moderate response was seen over sebaceous glands.

The thin *transitional* epithelium of kidney pelvis showed a number of foci, while the thicker epithelium of the bladder produced only an occasional reaction.

In the testes, which were not fully mature, the *seminiferous* epithelium at 8 hours showed a variable number of reaction foci confined to cells proximal to the basement membrane (Fig. 19). When only one or two foci were present per tubular cross section (lower center, Fig. 19), they were over the few spermatogonia present at the beginning of spermatogenesis (at the period of the cycle referred to as stages IX–XIV in [15]). As spermatogonia became

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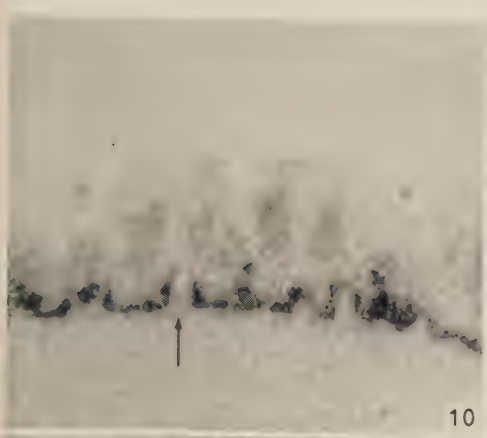
All pictures are radioautographs of H & E stained, RNAse-treated tissues from 12-g mice given an injection of adenine-C^{14}. Exposure 2–4 months.

Figs. 10 and 11.—Epithelium of tongue at 24 and 72 hours. At 24 hours (*Fig. 10*) the reaction predominates along the basal layer of epithelial cells, with the greatest intensity over the cells underlying each epithelial papilla, and the least over those in the interpapillary portions of the epithelium (arrow). At 72 hours (*Fig. 11*), the reaction zone was nearer the epithelial surface in the areas under than in those between papillae (arrow). × 214.

Figs. 12 and 13.—Ascending colon, 8 and 72 hours after injection. The reaction at 8 hours (*Fig. 12*) is over cells in the lower half of the crypts, with a maximum about midway up the sides of the crypts. By 72 hours (*Fig. 13*), the maximum intensity is over the epithelial cells bordering the gut lumen. × 107.

Figs. 14 and 15.—Descending colon, 8 and 72 hours after injection. At 8 hours (*Fig. 14*), the reaction overlays cells in the lowest third of the crypts. The U-shaped or circular pattern of the reaction is due to the bulk of the radioactivity being located in the nuclear region of the epithelial cells and not in the mucous portions of these cells nor in the lamina propria. At 72 hours (*Fig. 15*), the reaction maximum is at the mouths of the crypts (arrows). × 107.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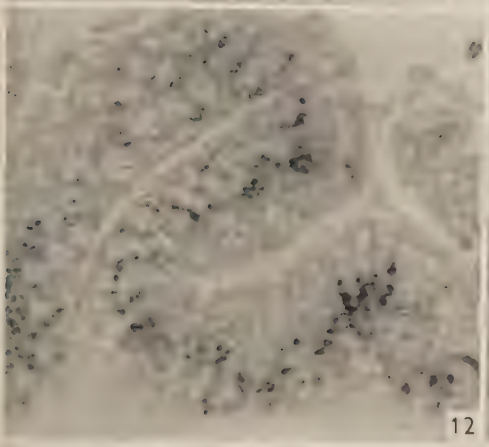
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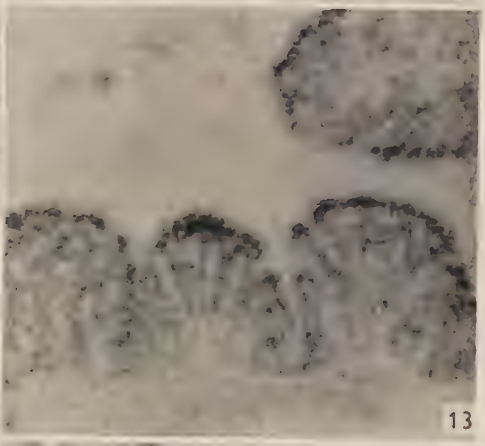
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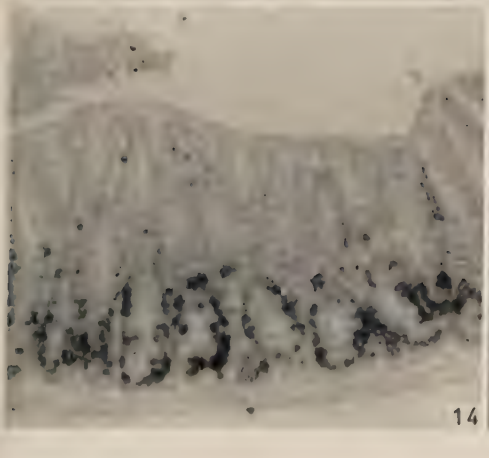
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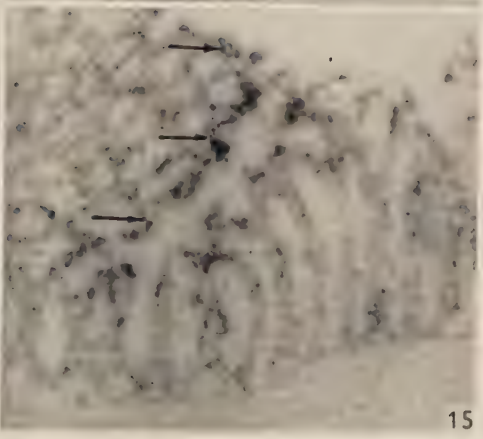
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more numerous, the reaction foci also became more frequent (stages I–V). Finally, a ring of foci was found at the periphery of the tubule (left of center, Fig. 19), in which case the reaction was over last generation spermatogonia (stage VI) or the young, so-called resting spermatocytes present at stage VIII. No reactions were observed over more advanced spermatocytes or spermatids. At 24 and 72 hours reactions appeared over leptotene and zygotene spermatocytes at stages IX–XII (Fig. 20).

Hemopoietic tissues.—Intense radioautographs characterized spleen, thymus (Fig. 2, top), lymph nodes and bone marrow at 8 hours. At 24 hours, there was little change in pattern, except for a slight decrease in intensity (Fig. 2, middle). At 72 hours, definite signs of radiation damage were seen in these organs and, therefore, any conclusions reached for that period can only be tentative. In the *spleen* at 8 hours, the lymphatic nodules of the white pulp displayed an intense reaction over germinal centers (see the four dark spots across the middle of Fig. 7), but no reaction over the cortex except for scattered small foci corresponding to individual lymphocytes. The reaction over germinal centers was less intense at 24 hours (Fig. 8), while at 72 hours only widely scattered radioactive foci could be seen (Fig. 9). In the red pulp (Fig. 7), unreactive zones corresponding to typical sinuses and cords alternated with intensely reactive zones corresponding to sinuses filled with groups of bone marrow-like cells (myeloblasts, erythroblasts, etc.). There were a number of megacaryocytes, only a few of which were overlaid by

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Radioautographs of RNAse-treated tissues from mice given an injection of adenine-C^{14}.

Fig. 16.—H & E stained radioautograph of the body of the stomach 8 hours after injection. 4-month exposure. The foci corresponding to radioactive cells are in the isthmus region of the mucosal glands. × 107.

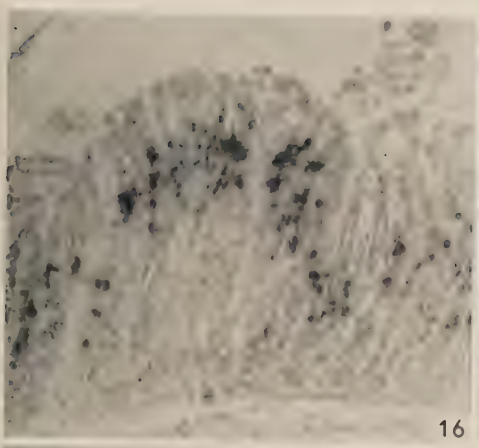
Fig. 17.—H & E stained radioautograph of duodenum, 24 hours after injection. 4-month exposure. The maximum of the reaction is somewhat higher up the sides of the villi than in the jejunum at the same time interval (compare with Fig. 5). × 107.

Fig. 18.—H & E stained radioautograph of ileum, 8 hours after injection. 2-month exposure. A strong reaction can be seen over the crypts. × 107.

Fig. 19.—Unstained radioautograph of testis in 12-g mouse, 72 hours after injection. 6-month exposure. Many tubules show a ring of reaction foci near the basement membrane. Several tubules show a few small, widely separated, reactions near the basement membrane. A tubule in the lower center shows only one reactive focus. (Note that both intense and weak reaction foci may be seen.) × 214.

Fig. 20.—H & E stained radioautograph of testis tubules in 15-g mouse, 72 hours after injection. 2-month exposure. The tubule crossing the center of the photo shows many reaction foci due to spermatocytes at the leptotene stage. (The larger diameter of the tubules in this picture than in Fig. 19 was due to a more advanced stage of development of the testis in this animal.) × 107.

Fig. 21.—H & E stained radioautograph of skin, 24 hours after injection. 2-month exposure. Hair follicles display a strong reaction over their base and the proximal part of the shaft. × 53.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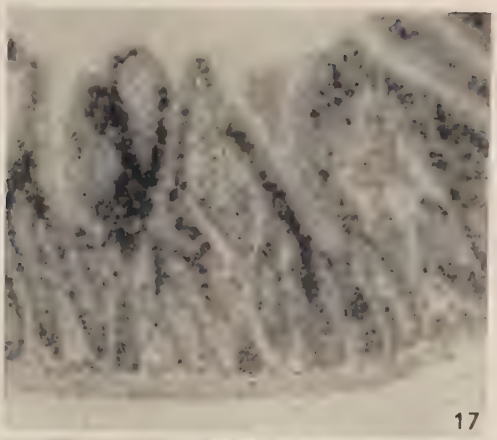
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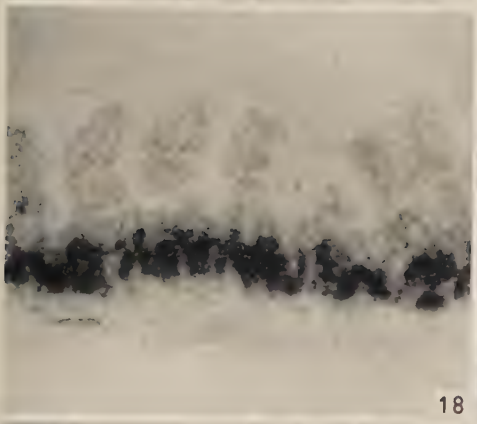
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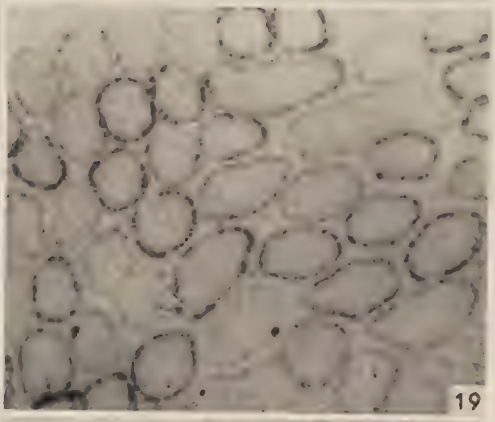
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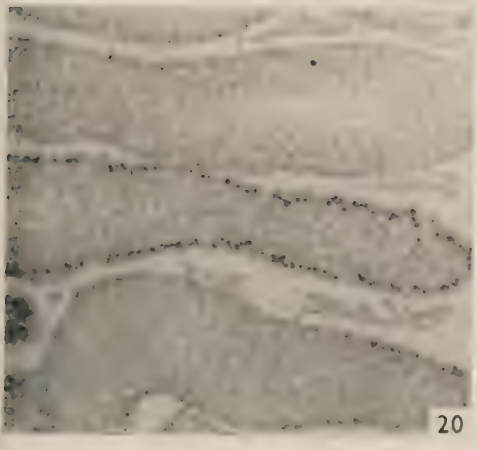
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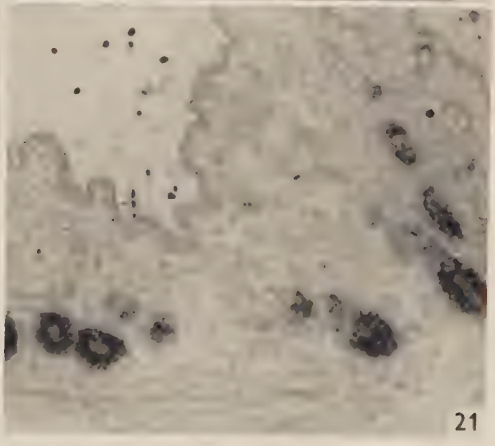
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silver granules. In *lymph nodes*, as in the white pulp of the spleen, an intense reaction was seen over the germinal centers of the lymphatic nodules with scattered foci elsewhere. The *thymic lobules* at 8 hours displayed a strong reaction over the cortex and a weak one over the medulla (Fig. 2, top). Under high power, small reactive foci were seen to be scattered in the medulla, whereas in the cortex the reactions were gathered into more or less distinct masses. In the lumen of *blood vessels* a few white blood cells showed a reaction at 24 hours. By 72 hours their number had increased and, although no counts were made, it was apparent that a few of the lymphocytes and many of the neutrophiles were overlaid by a reaction (Fig. 26, left center).

Connective tissue and derivatives.—Individual foci corresponding to connective tissue cells were fairly common in laminae propriae (e.g. bladder), dermis and hypodermis, adipose tissue, etc. The frequency of the foci varied greatly with the location. For instance, the right half of the field of the lamina propria of the trachea reproduced in Fig. 25 contains about ten radioactive cells. The connective tissue layer lining the upper surface of the dentin of the lower incisor (periodontal membrane) showed numerous foci of reaction, which were especially crowded near the tips of the roots (Fig. 22,

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H & E stained radioautographs of RNAse-treated tissues from mice given an injection of adenine-C^{14}.

Fig. 22.—Basal portion of a lower incisor tooth, 24 hours after injection. 4-month exposure. From below upwards, note the pulp (*P*), the odontoblast layer (*O*), an artefactual space, dentin (*D*), a strip of connective tissue cells regularly arranged to form the periodontal membrane (*Ct*), a blood vessel (*Bv*) and alveolar bone. The connective tissue strip is crossed by a dark band resulting from the presence of a number of reaction foci. × 214.

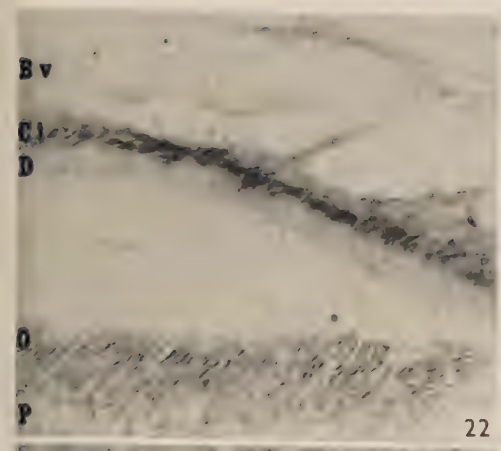
Fig. 23.—Basal portion of a lower incisor tooth immediately below that shown in Fig. 22. 3-month exposure. From the top downwards, note the pulp with the odontoblasts (*O*), dentin (*D*), the ameloblast layer (*A*), stratum intermedium (*SI*) and stellate reticulum (*Sr*). The ameloblast nuclei on the left produce no reaction, but on the right a number of them darken the emulsion. The juxtaposed portion of the stratum intermedium also shows reaction foci. × 214.

Fig. 24.—Cerebellum, 24 hours after injection. 2-month exposure. Grey matter (granular layer) crosses the top of the field. The remaining material is white matter, which contains one cell overlaid by silver granules. × 445.

Fig. 25.—Connective tissue in the lamina propria of the trachea, 72 hours after injection. 4-month exposure. Most of the field is occupied by connective tissue. The tracheal cartilage is in the upper center. A number of individual, scattered reactions can be seen over connective tissue cells on the right-hand side. × 445.

Fig. 26.—Lung tissue, 72 hours after injection. 4-month exposure. The left third of the picture is occupied by a blood vessel in which there is a radioactive leucocyte. On the right, an alveolar wall displays two reaction foci. × 445.

Fig. 27.—Cartilage from the epiphyseal plate of a long bone, at 72 hours after injection. Serially arranged cartilage cells extending diagonally from upper left to lower right show several reaction foci. No foci are seen over the hypertrophied cells at lower left. × 445.



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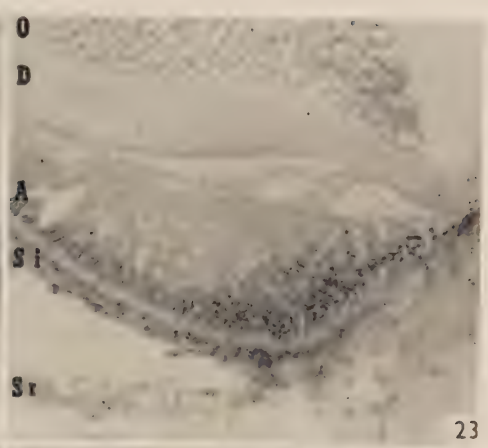

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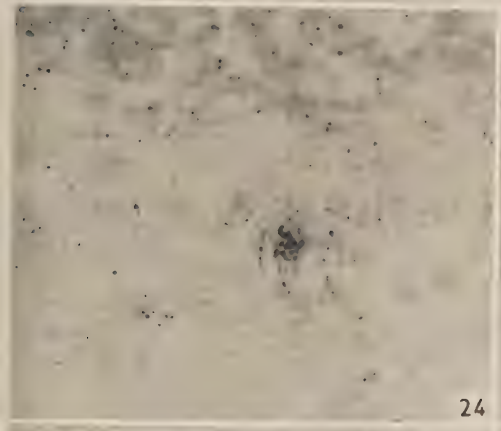

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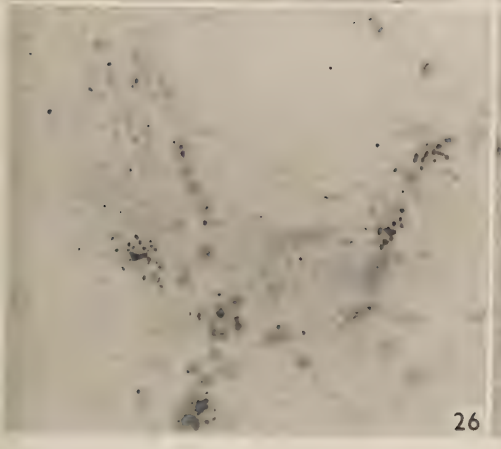

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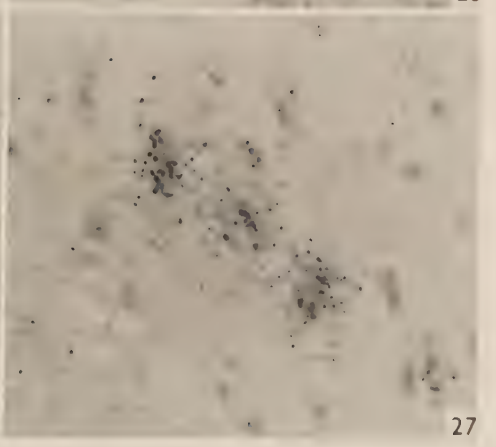

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Ct). A smaller number of foci was observed in the alveolar walls of the lung (Fig. 26) and was attributed to the so-called alveolar cells, which may be considered to be the connective tissue cells of this tissue [4]. It should be emphasized that, particularly in the lamina propria of the intestine (see the foci within the villus on the extreme left of Fig. 6), it was difficult to decide whether the responses were over true connective tissue cells (fibrocytes, fibroblasts) or migrating cells (lymphocytes, etc.). Reactions were occasionally seen over the striated *muscle* around the tibia and over cardiac muscle, but many if not all of these were attributable to interstitial connective tissue cells.

In general, cartilage, bones and teeth were unreactive. However, the *cartilage* of the epiphyseal plate showed a number of reaction foci over the zone of proliferating cells (Fig. 27, center) but none over the zone of hypertrophic cells (Fig. 27, lower left). Also, the root tip of the continuously growing *incisor teeth* showed intense reactions over: (1) many cells of the periodontal membrane as mentioned above (Fig. 22, *Ct*); (2) some odontoblasts and pulp cells; and (3) many ameloblasts (Fig. 23). The juxtaposed portion of the stratum intermedium also showed reactive foci.

Other tissues.—Liver, pancreas, salivary (submaxillary and sublingual) glands (Fig. 2), adrenal and thyroid typically exhibited rare scattered foci with a seemingly random distribution. The frequency of these foci seemed to be greater in the salivaries than other glands.

The grey matter of cerebrum and cerebellum was generally non-reactive, but the occasional focus was seen in the white matter (Fig. 24).

Ribonuclease-labile Radioactivity

The location and intensity of the labelled material removed by treatment with ribonuclease was deduced from the comparison of control (Fig. 1) and ribonuclease-treated slides (Fig. 2). Two kinds of sites contained such labile material:

(1) The same sites which displayed the ribonuclease-stable material described above. Thus, the presence of labile radioactivity in intestine, colon, spleen, thymus and testis was shown by the lesser intensity of the reactions in Fig. 2 than in Fig. 1.

The superimposition of the patterns for labile and stable material was closest in hemopoietic tissues. In the intestine at 8 hours, the labile radioactivity was strong over both nuclei and cytoplasm of the cells of the crypts, and was moderate over the villus epithelium with a decreasing gradient from the base to the tips. In stratified epithelia (gum, epidermis) and also in hair

follicles, not only the basal layer, but most of the malpighian layers contained such material, with a predominance below the papillae in the case of the tongue. The transitional epithelium of bladder showed labile material throughout. Thus, in these tissues the ribonuclease-labile radioactivity was present in many more cells than the stable radioactivity.

(2) In those tissues with few or no foci produced by ribonuclease-stable radioactivity, the labile radioactivity was occasionally abundant. This was the case in liver and salivaries and, to a lesser extent, pancreas and adrenals. In the cerebrum, cerebellum and retina, ribonuclease-labile material was found in several areas with a dense cell population.

Subsidiary Experiments

Adenine-C^{14} radioautographs of newborn mouse tissues.—The distribution of ribonuclease-stable radioactivity in newborn mice had many similarities with that in 12–15 g mice. Thus, strong reactions were seen at 8 hours over the rudimentary crypts of the small intestine, basal cells of the epidermis, base of hair follicles, hemopoietic tissues, etc.

Weakly reactive tissues (pancreas, submaxillary, heart muscle, lung) also showed widely scattered foci and a weak background reaction as in 12–15 g mice. However, moderate to strong radioautographic reactions were seen in certain areas that displayed little or no reaction in the older mice, namely, the kidney cortex, the whole of the cartilage in tibia and femur, striated muscle fibre nuclei in leg muscle, and smooth muscle of the intestine.

Thymidine-C^{14} radioautographs of tissues from a 12-g mouse.—The reactions, which were observed only in one animal sacrificed at 24 hours after injection (Fig. 3), were intense over spleen, thymus, small intestine, and colon, moderate over testis, weak over salivary glands, and absent over liver. The arrangement of the reactions in the form of small foci which merged into solid masses when the radioactive cells were closely packed, as well as the detailed pattern in all tissues studied, was identical with that of ribonuclease-stable material at 24 hours after adenine-C^{14} injection (Fig. 2). However, the diffuse background reaction seen with labelled adenine, particularly over liver (Fig. 2), was not observed with labelled thymidine (Fig. 3).

DISCUSSION

The radioautographic reactions observed 8 hours after adenine-C^{14} injection had a reproducible distribution and could be assigned to definite structures. The only exception was noted in the kidney, where dense reaction centers

were scattered over the sections without any obvious relation to structure. These reactions may be due to an adenine metabolite in the process of being excreted, perhaps the 2,8-dioxyadenine detected in the urine of animals to which adenine had been administered [3]. In contrast, the well localized, reproducible reactions observed in other organs and tissues must be caused by insoluble adenine metabolites held in tissue structures.

At the 24- and 72-hour intervals, a reproducible pattern was also obtained except again for kidney and, in addition, hemopoietic tissues. In the latter case, the irregularities may be explained by our finding of signs of radiation damage, particularly at 72 hours, with the doses around 1 μc adenine-C^{14} per gram body weight used in this work (as well as by others [24, 28]). Another difficulty encountered in the course of this investigation arose from the fact that the amount of adenine administered was high enough to produce signs of toxicity soon after injection, especially the large dose used in the second series. Even so, the similarity of the results in the first two series and the regularity of the patterns observed (except in kidney and hemopoietic tissues) were taken to indicate that reliable results had been obtained.

The regular radioautographic reactions observed in most cases were presumably caused by one or several of the labelled substances present in tissues after administration of adenine-C^{14} to rats, that is: (*a*) *adenine* itself, which, however, cannot be involved since it is rapidly eliminated from the body and disappears in less than two hours [3]; (*b*) *5′-adenylic acid derivatives*, the specific activity of which reaches a maximum within two hours after administration and decreases slowly over the next few days or weeks [3]; but unpublished work (B. Messier and B. E. Walker) indicated that these nucleotides were not retained in histological sections after Carnoy fixation (although they were when mercury salt-containing fixatives were used) and, therefore, they could not be responsible for the radioautographic reactions observed here; (*c*) *the nucleic acids, RNA and DNA*, which have a specific activity reaching a maximum after 24 48 hours [3] and are retained in sections after Carnoy fixation [33], so that they may be held responsible for the radioautographic reactions described in the present work.[1]

In the hope of distinguishing between the two labelled nucleic acids present in the sections, an attempt was made to remove RNA by treatment with a ribonuclease preparation. Radioautographs obtained with (Fig. 2) or without (Fig. 1) pretreatment with ribonuclease were then compared.

[1] Consideration of specific activities [3] indicated that, after administration of labelled adenine, this substance is first taken up into the 5′-adenylic acid fraction, which in turn yields the guanine and adenine residues built into newly formed nucleic acids [6, 8, 29]. This sequence occurs without change in the adenine ring structure [1].

Nature of Ribonuclease-stable Radioactivity

That the ribonuclease-stable radioactivity consisted of DNA was confirmed by its location in the nuclei of intestinal epithelial cells (Fig. 5), spermatocytes (Fig. 20), ameloblasts (Fig. 23), etc., as well as by its complete removal with hot trichloracetic acid. The light background reaction observed in many locations, particularly liver (Fig. 2), may be due to the persistence of some RNA as a result of the low effectiveness of ribonuclease treatment in some tissues [33] or perhaps to retention of RNA fragments [27]. If these background reactions are ignored, then the distribution of ribonuclease-stable radioactivity at 24 hours after adenine-C^{14} injection (Fig. 2, middle) was identical to that observed after thymidine-C^{14} injection (Fig. 3). Accordingly, since it is known that thymidine is incorporated only into new DNA [8, 10], ribonuclease-treated adenine radioautographs must also reveal new DNA.

The synthesis of new DNA occurs only at the time of the duplication of chromosomic material which takes place before mitosis (reviewed in [30]). (The other possible result of DNA synthesis is endopolyploidy, but this condition has not been seen with certainty in mammals except in some cancer cells [20].) The radioactivity seen over some mitoses in the intestinal crypts at 8 hours after adenine-C^{14} injection illustrated the occurrence of DNA synthesis in these cells at some time during the 8-hour interval. Since mitosis gives rise to new cells—only exceptionally to binucleate cells—it may be concluded that the DNA synthesis visualized in the radioautographs indicates the formation of new cells.

Since cells do not necessarily stay at their formation site, the earliest radioautographs would be the most likely to catch new cells at or near the place where they arise. Accordingly, the 8-hour reactions were taken to provide the best indication of the *sites of formation of new cells.*

Comparison of the three parts of Fig. 2 indicated that the reactions observed over spleen, thymus and intestine reached a maximum at 8 hours. Hence the bulk of the labelling must have occurred during the 8 hours following injection and any labelling occurring later can only be slight. However, it was apparent in some tissues, such as oesophagus, tongue and testis that, in addition to the intensely labelled cells formed early, a number of weakly labelled cells appeared at 24 and 72 hours—a fact in agreement with chemical data showing an increase in the specific activity of DNA up to 24–48 hours after adenine-C^{14} injection [3]. Furthermore, if the intensely labelled cells are traced at the 3 time intervals in various organs, it is possible to determine whether the new cells remain *in situ* or *migrate away from their formation site.*

Sites of Formation and Migration Pathways of New Cells

Gastro-intestinal epithelia. –The difference in the location of the reactions at the three time intervals in gastro-intestinal epithelia pointed to a migration of the component cells. Thus, in *small intestine*, the sequence of events was that new cells form in the crypts (8-hour interval, Figs. 4 and 18), migrate up the sides of the villi (24-hour interval, Fig. 5) and reach the villus tips as early as 24 hours in the duodenum (Fig. 17) and somewhat later in jejunum and ileum (Fig. 6). The lesser intensity of the reaction at 72 than at 24 hours in jejunum (compare Figs. 5 and 6) suggested that by 72 hours the most reactive cells had already been lost from the villus tips to the lumen (17). In the *colon*, where cells form in the crypts distal to the gut lumen (Figs. 12 and 14) and then move towards it (Figs. 13 and 15), a few radioactive cells were indeed found free in the lumen at 72 hours. Finally, in both the pyloric portion and the body of the *stomach*, new epithelial cells arose in the isthmus of the glands (Fig. 16) and migrated along the walls of the pits towards the lumen.

Thus, a continuous flow of new cells ascends intestinal villi and colonic crypts as well as gastric pits to eventually fall into the lumen, so that the whole gastro-intestinal lining is involved in a massive renewal. In the past, the existence of such renewal was deduced from mitotic counts and morphological features in both intestine [17] and stomach [31]. Attempts to visualize these phenomena by radioautography have met with some success [12, 16, 18, 25]. However, the present comprehensive series of pictures illustrating the pathway of cell migration in small intestine (Figs. 4–6), colon (Figs. 12–15) and stomach (Fig. 16) leaves no doubt as to the existence and speed of the epithelial renewal in the digestive tract. This renewal insures the integrity of the gastro-intestinal lining in spite of the presence of enzymes, bacteria and other noxious agents in the lumen, as the continuous inflow of new cells is forever anticipating damage.

Other types of epithelia.—Over the 72-hour period, most *stratified squamous epithelia*, including most regions of the gum, showed little displacement of the few cells formed in the basal layer by 8 and 24 hours. However, in the periodontal portion of the gum, labelled cells displayed evidence of migration, since by 72 hours some of them approached the surface. Thus, the cells migrate somewhat independently. Such a conclusion was not reached in radioautographic studies of epithelia, using the protein precursors bicarbonate-C^{14} [12] and methionine-S^{35} [16], presumably because the simultaneous

protein synthesis by all deep malpighian cells gave the impression that a solid layer was labelled and moved outward en masse.

In the tongue epithelium, the malpighian cells corresponding to the epithelial papillae were formed at a faster rate (Fig. 10) and progessed lumenward more rapidly (Fig. 11) than the interpapillary cells (Figs. 10 and 11, arrows). Thus the papillae must be renewed more swiftly than the intervening epithelium.

In the *seminiferous epithelium* at 8 hours, spermatogonia and young primary (so-called resting) spermatocytes were radioactive. By 24 and 72 hours, radioactivity was found in leptotene and zygotene spermatocytes (Fig. 20) presumably as a result of the evolution of the resting spermatocytes labelled at the early time interval. It may be noted that the DNA synthesis occurring in resting spermatocytes was not followed by mitosis as usual, but by leptotene and subsequent stages of meiosis, which extend over about two weeks before yielding secondary spermatocytes and spermatids [15]. Such a long time interval between DNA synthesis and the formation of new cells (already implied in previous observations [32]) is unique.

Hemopoietic tissues.—The 8-hour results supplied information on cell production in lymphatic and myeloid tissue. In the lymphatic nodules of spleen and lymph nodes, massive cell formation was taking place in germinal centers, with only scattered cells being produced in the cortex (Fig. 7). Conversely, in the thymus, the cortex exhibited many intense reaction masses, which were attributed to large numbers of proliferating cells, whereas the medulla showed only scattered cells undertaking DNA synthesis. The crowded, intense reactions observed in the myeloid tissue of spleen (Fig. 7) and bone marrow also revealed the presence of many islands of rapidly proliferating cells.

Although caution is required in interpreting the observations made at 24 and 72 hours in view of the radiation damage seen at the last time interval, the progressive decrease in the radioactivity of spleen (Figs. 7–9) and other hemopoietic tissues with time suggested the release of newly formed blood cells to the circulation. This conclusion is supported by the observation of a few labelled lymphocytes and many labelled neutrophiles in the lumen of blood vessels at 72 hours (Fig. 26).

Connective tissue and derivatives.—One of the prominent results of this investigation is the demonstration of active cell production in connective tissue. So far, a fair rate of mitosis had been found in alveolar cells—the connective tissue cells of the lung [4, 5]—but it was felt, perhaps wrongly, that the ability to divide was related to the differentiation of fibroblasts into

alveolar cells in the lung [4]. Recently the mitotic index of skin connective tissue cells was found to be as high as that of epidermal cells in the rat [9]. Since the present investigation revealed that cell formation was fairly common not only in loose connective tissue, but also in the more specialized Kupffer cells and fat cells, the mitoses reported for alveolar and dermal cells may only be local manifestations of a widespread tendency of connective tissue cells to proliferate.

Whereas cell production is compensated by a loss of cells to the alveolar spaces in the lung [4], it is likely that the cell formation occurring in other areas of connective tissue is also balanced by an equivalent cell loss. Whether such loss of connective tissue cells is due to death, migration to the outside or transformation into other cell types (e.g. monocytes) is not known. In any event, connective tissue may have to be added to the list of "cell renewal systems" [19].

There were considerable differences in the rate of cell production in various areas of connective tissue and, therefore, local conditions may influence this phenomenon. An outstanding example is the active cell multiplication present in the periodontal membrane of the incisor tooth, particularly in the region of the root tip (Fig. 22). In this case, the new connective tissue cells may replace others which are carried away with the growing tooth.

With regard to the tissues derived from, or associated with connective tissue, examination of *teeth* confirmed that the production of odontoblasts and ameloblasts is confined to the root tip. *Cartilage* in 12–15 g mice showed no signs of cell production, except in the epiphyseal plate of long bones (Fig. 27). In contrast, in newborn mice, new cells appeared throughout the cartilage which makes up the epiphysis, a fact indicating interstitial rather than perichondral growth. Similarly, striated and smooth *muscle* fibers had many reactive nuclei in newborn mice, whereas in 12–15 g mice the few labelled nuclei seen were those of interstitial connective tissue cells.

Other tissues.—Other tissues, such as liver, pancreas and adrenal, showed only infrequent scattered foci with no evidence of cell migration. The number of foci was largest in salivary glands (submaxillary and sublingual) and smallest in cerebrum and cerebellum.

Classification of the sites of cell formation.—The cell populations of the adult organism have been classified on the basis of mitotic activity into three groups [19]: those with no mitoses, those with infrequent mitoses (attributable to the slight growth of adult animals), and those with abundant mitoses (cell populations undergoing renewal). The first or non-mitotic group included cerebrum and cerebellum, which in our 12–15 g mice were found to show

only rare radioautographic reactions. The second group—tissues with infrequent mitoses such as liver, pancreas, adrenal cortex, and thyroid—produced scattered reaction foci of low frequency, probably because the animals used had a low growth rate. The third group—cell populations with abundant mitoses, such as gastro-intestinal epithelia, other epithelia (epidermis, gum, testis) and blood cell-forming organs—produced moderate to intense radioautographs. Thus in general, radioautographic results supported the classification of cell populations into three main groups.

In newborn mice too, renewing tissues like epidermis and hemopoietic organs (spleen, thymus, bone marrow) were much more radioactive than organs like pancreas, submaxillary and heart muscle, so the cell renewal systems are already in production at this early age. Only tissues undergoing unusual spurts of growth in the newborn, like kidney cortex and the cartilage and muscle of leg, showed a concentration of radioactivity approaching that of cell renewal systems. The fact that radioactivity was greater in cell renewal systems than in tissues undergoing only growth, even in an animal developing as rapidly as the newborn mouse, emphasized the prominent part played by cell renewal systems in the total DNA synthesis of mammals.

Nature and Distribution of Ribonuclease-Labile Radioactivity

Since ribonuclease removed the cytoplasmic basophilia (liver, pancreas, nervous tissue) which was shown by spectrophotometric and other means to consist of RNA [33], it may be concluded that the ribonuclease-labile radioactivity was composed of newly formed RNA. New RNA arises in two kinds of locations:

(1) The sites of cell formation. The abundance of newly formed RNA in newly formed cells indicated that, in dividing cells, new RNA was synthesized as well as new DNA. Furthermore, the presence of new RNA at 8 hours in the post-mitotic cells at the bases of the intestinal villi and in the malpighian layer of epithelia suggested that RNA synthesis continues for some time after completion of mitosis.

(2) Certain organs with stable cells such as liver, pancreas, adrenal cortex, some parts of the nervous system and some other tissues (transitional epithelia). The RNA synthesis occurring in these sites may indicate the existence of a continuous RNA turnover.

It is interesting, in view of current theories, that there is a rough parallel between the various sites of RNA synthesis and those of protein synthesis as revealed by radioautography after administration of methionine-S^{35} [16].

SUMMARY

A detailed radioautographic study was made of tissues taken from 12–15 g mice at 8, 24 and 72 hours after adenine-8-C^{14} injection. Newborn mice killed 8 hours after adenine-C^{14} injection and a 12-g mouse killed 24 hours after thymidine-C^{14} injection were also studied. The sites of incorporation af radioactivity after adenine-C^{14} injection were studied in radioautographs of slides with or without ribonuclease treatment.

The reaction foci seen in ribonuclease-treated slides were attributed to *newly synthesized DNA* because they were seen over nuclei and mitoses, they were removed by extraction with hot trichloracetic acid, and they were similar in distribution to the foci seen after injection of thymidine-C^{14}, a specific precursor of DNA. The location of the new DNA at the early interval was in fact the location of the cell production sites. At later intervals, however, the new DNA could be seen at some distance from the production sites, thus indicating the migration pathway of the newly formed cells.

The chief sites of cell production are:

(1) the epithelia of the digestive tract, where cells formed in the isthmus of gastric glands and in intestinal crypts migrate *en masse* towards and into the gut lumen, which they reach in about 2 days in the case of the duodenum and about 3 days for other parts of the gastro-intestinal tract;

(2) other epithelia (stratified, seminiferous, etc.) which show a variable rate of cell formation and migration to the lumen;

(3) the germinal centers of lymphatic nodules in lymph nodes and the white pulp of the spleen, as well as the cortex of thymus, and the hemopoietic cords of the red bone marrow and the red pulp of the spleen, which yield blood cells to the circulation as shown by the appearance of radioactive leucocytes in the blood stream;

(4) many types of connective tissue, such as loose connective tissue cells, fat cells, alveolar cells of lung, Kupffer cells of liver, periodontal cells of incisor tooth.

The tissues with high cell production, except perhaps for connective tissue, are those known to be composed of cell populations undergoing renewal [19]. The other tissues of 12–15 g mice (parenchymal cells of liver, adrenal, submaxillary, etc.) show only infrequent formation of new cells, probably contributing to the low growth rate of the animals. Even in newborn mice, cell production is predominantly in renewal systems, although it is also

active in certain areas undergoing a rapid burst of growth, such as kidney cortex, and the cartilage and muscles of leg.

The ribonuclease-labile radioactivity was attributed to *newly synthesized RNA*, which was found in (1) the sites of new cell formation listed above, so that both nucleic acids are synthesized in new cells; (2) some of the tissues with a relatively stable cell population, such as liver, pancreas, adrenal cortex, and nerve cells, where presumably RNA is being turned over.

This work was supported by a grant of the National Cancer Institute of Canada. The sample of thymidine-C^{14} was a gift from Dr. Morris E. Friedkin.

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TRANSPLANTATION OF NUCLEI BETWEEN *RANA PIPIENS* AND *RANA SYLVATICA*

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Received September 10, 1957

Rana pipiens and *Rana sylvatica* are two species of frogs that have an extensive geographic distribution in North America. A taxonomist would not regard them as being very closely related, and the limited genetic data available substantiate this opinion.

A variety of abnormal combinations of nuclei and cytoplasms of these two species is possible. The combinations that can be made are as follows:

(1) The cross *pipiens* ♀ × *sylvatica* ♂ produces diploid hybrids that develop normally to the beginning of gastrulation. Thereafter the embryos undergo a characteristic series of abnormal changes, but most never go beyond an early gastrula stage [7]. The embryos remain alive as arrested gastrulae for a long period of time, until the control embryos (*pipiens* ♀ × *pipiens* ♂) that began their development at the same time are in stage 20, or even older.

(2) The reciprocal diploid hybrids, *sylvatica* ♀ × *pipiens* ♂ develop normally to the beginning of gastrulation. The period of time that they remain alive as arrested gastrulae is short. Cytolysis occurs when the controls (*sylvatica* ♀ × *sylvatica* ♂) that began their development at the same time are at the end of gastrulation [6].

(3) The androgenetic haploid hybrid, formed by the fertilization with *sylvatica* sperm of a *pipiens* ovum, which has had its nucleus removed, develops normally to a late blastula stage. Embryos of this class remain alive as arrested blastulae until *pipiens* ♀ × *pipiens* ♂ controls are in stage 21 [9, 12].

(4) The reciprocal androgenetic haploid hybrid formed by the fertilization with *pipiens* sperm of a *sylvatica* ovum, which has had its nucleus removed, also develops normally to a late blastula stage. In common with the corresponding diploid hybrids (*sylvatica* ♂ × *pipiens* ♂), the haploid hybrids do not remain alive for long as arrested blastulae.

In this paper additonal data on the general topic of interrelations between the nuclei and cytoplasms of these two species will be presented. The experiments are of three sorts:

(1) The determination of cleavage rates of diploid hybrids and androgenetic hybrids.

(2) The transplantation of a diploid nucleus from one species to the enucleated cytoplasm of the other.

(3) The transplantation of a nucleus from embryos of the sort just mentioned (2) back to enucleated ovum of the species from which the nucleus was derived orginally.

MATERIALS AND METHODS

The adult *Rana pipiens* came from northern Vermont. The standard methods of obtaining eggs by pituitary injections and sperm by cutting up the testes in spring water (or 10 per cent Holtfreter solution) were used. The *Rana sylvatica* adults and embryos were collected locally during this species' breeding season. The females, consequently, had eggs in the uteri.

Porter's [10] method was used for making androgenetic embryos.

The method of King and Briggs [3] was used for transplanting nuclei, except that trypsin was not used to separate the cells. The donor nuclei were taken from the inner side of the blastocoel roof of stage 8, 9, or 10. I am greatly indebted to Briggs and King for instructing me in the techniques of their method for transplanting nuclei.

A constant-temperature water bath was used in those experiments where the cleavage rate was measured. In other experiments the embryos were kept in constant-temperature incubators at 10°, 15°, or 20°. All temperatures used were well within the normal range for both species.

The following abbreviations will be used: *syl* for *Rana sylvatica* and *pip* for *Rana pipiens*. In listing crosses the female species will be given first. In the case of androgenetic embryos, the species from which the spindle was removed will be indicated in the conventional manner, (*pip*) or (*syl*).

Cleavage Rates in Diploid Hybrids and Androgenetic Hybrids

In general the embryonic development of *Rana sylvatica* proceeds at a faster rate than that of *Rana pipiens* [5]. This difference is not reflected in the cleavage stages, the two species being nearly identical. Since there is no difference between the maternal and paternal rate of cleavage, the only information that can be derived from hybrid combinations is whether or not the combinations cleave at a normal rate. This information is of considerable interest in the case of androgenetic combinations.

There have been several previous reports on cleavage rate in some of the possible combinations. Data for cleavage rate to the 16-cell stage in the *syl* × *pip* hybrids are given by Moore [6]. In the reciprocal hybrid, *pip* × *syl* it was reported [7] that cleavage was normal but data on rate were not given. Ting [12] and Moore and Moore [9] report that early development is normal

TABLE I. *Rate of cleavage in diploid and haploid* Rana pipiens *and* Rana sylvatica, *and in the diploid and haploid hybrids.*

Interval between cleavages in minutes.

Exp.	Temp.	Combination	Fert. to 1st cl.	1st cl. to 2nd cl.	2nd cl. to 3rd cl.
4/7/56	20.1°	*syl* × *syl*	141	46	45
		syl × *pip*	145	49	46
4/8/56	20.3°	*syl* × *syl*	137	52	49
		syl × *pip*	150	49	52
		pip × *pip*	137	52	51
		pip × *syl*	137	52	51
4/9/56	20.2°	*syl* × *syl*	143	45	45
		syl × *pip*	143	51	44
3/17/57	20.2°	*syl* × *pip*	139	47	45
		(*syl*) × *pip*	143	46	45
3/18/57	20.2°	*syl* × *syl*	145	45	45
		(*syl*) × *syl*	145	45	45
3/27/57A	20.2°	*pip* × *syl*	135	49	51
		(*pip*) × *syl*	135	50	50
3/27/57B	20.2°	*pip* × *syl*	139	53	—
		(*pip*) × *syl*	140	52	—
4/7/57	20.3°	*pip* × *pip*	134	50	48
		(*pip*) × *pip*	134	50	48
		pip × *syl*	131	49	49
		(*pip*) × *syl*	131	50	48
4/11/57	20.4°	*pip* × *pip*	138	51	52
		(*pip*) × *pip*	139	51	52
		pip × *syl*	138	50	52
		(*pip*) × *syl*	138	50	51

in the androgenetic hybrid (*pip*) × *syl*. In neither case, however, were data on cleavage rate given.

Nine experiments were performed to measure the rate of cleavage of the various possible combinations of nuclei and cytoplasm. The data are given in Table I.

The following points will aid the interpretation of the data.

In those experiments that involve only diploid embryos, for example

4/8/56, the testes of a single male of each species is used for the sperm suspension. The ova are stripped from the female, fertilized, and placed in the constant-temperature water bath. All groups are subjected to the same temperature throughout.

In those experiments involving two groups of haploid embryos, such as 4/7/57, the two groups may not be at the same temperature for the first 40 minutes after fertilization. This is a result of the necessity of keeping the embryos at room temperature when the maturation spindle is being removed. An attempt was made to keep the room temperature as constant as possible, and the embryos at 19 20° before they were put into the constant-temperature bath. Each group of haploids, however, is in the same dish as their diploid controls, so the temperature will be the same for both. In the experiment cited as an example, no significance should be attached to the fact that the *pip* × *pip* and (*pip*) × *pip* begin first cleavage in 134 minutes, and the *pip* × *syl* and (*pip*) × *syl* embryos in 131 minutes. Since the embryos were always put into the bath by 40 minutes after fertilization, a direct comparison can be made among the various groups of embryos for the intervals between cleavages.

The data of Table I show that there are slight differences in the rates of cleavage between various groups of eggs. In those experiments involving *pipiens* eggs, there is no deviation from the maternal rate in *pip* × *syl*, (*pip*) × *pip*, or (*pip*) × *syl* combinations. In experiments involving *sylvatica* eggs, a slight lengthening of the interval between fertilization and first cleavage and between first and second cleavages may occur in the *syl* × *pip* hybrids. If this difference is real, and I believe that it is, it is of short duration since the interval between second and third cleavage is equal to the maternal rate (except for the 4/8/56 experiment, but here the longer than normal second to third cleavage interval is balanced by a shorter than normal first to second cleavage interval).

We can conclude from these experiments that, apart from a possible lag in *pip* × *syl* hybrids, that the diploid hybrids, androgenetic haploid embryos, and androgenetic haploid hybrids cleave at the maternal rate. Thus the diploid hybrids and androgenetic hybrids cleave at the normal rate even though they have such poor developmental prospects.

The Transplantation of a Diploid Nucleus from one Species to the Enucleated Cytoplasm of the Other

The remarkable method developed by Briggs and King [1, 3] for the transplantation of embryonic cells to the enucleated cytoplasm of an uncleaved

ovum offers still another means for studying the relations of the nucleus of one species with the cytoplasm of another.

The essential features of their method are as follows. A cell, which is to be transplanted, is drawn into a pipette that has a bore smaller than the diameter of the cell. As a consequence the cell membrane is broken and the cytoplasm somewhat dispersed. The nucleus, however, remains intact. The intact nucleus surrounded by its disrupted cytoplasm is then injected into an ovum from which the second maturation division spindle, and hence all nuclear material, has been removed.

It is customary to speak of this operation as "transplantation of nuclei" and to ascribe the results to the action of the nucleus alone. The transplanted cytoplasm is assumed to have no effect. Briggs and King have given compelling reasons for believing these assumptions to be true.

When an experiment of this sort is performed using nuclei from the inner side of the blastocoel roof of a late blastula or early gastrula, it is found that the experimental embryo can develop into a fully formed tadpole. If one has the patience and desire, such an embryo may be raised to the juvenile stage. This indicates that the nuclei have not undergone irreversible differentiation by the time they were transplanted.

When the nucleus from the under side of the blastocoel roof of a late blastula or early gastrula of *sylvatica* is transplanted to an enucleated ovum of *pipiens* the resulting embryo develops to a late blastula stage. There is no indication of a dorsal lip in any embryo. In 42 operations of this sort these were the results:

> 12 were entirely normal and reached a late blastula stage; 10 formed partial blastulae (a term used to denote those embryos that have some external portion of the embryo undivided); and 20 failed to cleave normally.

In the controls for the operative procedure, involving the transplantation of *pipiens* blastula nuclei into enucleated *pipiens* ova, some embryos developed into normal tadpoles, but none were carried to metamorphosis. Thus, in the 50 operations performed immediately before those involving *pipiens* and *sylvatica* the following results were obtained:

> 16 produced normal larvae; 10 produced gastrulae or neurulae; 16 produced partial or complete blastulae; and 8 showed no cleavage or abortive cleavage.

The inability of a diploid *sylvatica* nucleus to collaborate with the enucleated *pipiens* cytoplasm in the production of a normal embryo is not surprising.

The fact that such a combination will develop only to the late blastula stage might have been anticipated since the same developmental end point was reached in the (*pip*) × *syl* haploid embryos. Neither a haploid nor a diploid set of *sylvatica* chromosomes is able to organize the materials of a mature *pipiens* cytoplasm in such a way as to get the embryo beyond gastrulation.

One can then ask the following question: "What is the nature of the chromosomes in these late blastulae that are produced when a diploid *sylvatica* nucleus is placed in *pipiens* cytoplasm?" In the course of development the chromosomes underwent about 15–16 mitotic divisions. Each time the *sylvatica* chromosomes replicated they did so from substances in the *pipiens* cytoplasm. Are they still *sylvatica* chromosomes when they reach the late blastula stage? This questions can be answered by transplanting these nuclei back to *sylvatica* cytoplasm. If the chromosomes are normal, the embryos so formed should develop as normal larvae. If not, we might expect abnormal development.

Transplantation of Nuclei from Interspecific Embryonic Combinations Back to the Cytoplasm of the Nuclear Species

If one attempts to conduct the experiment just outlined with *pipiens* and *sylvatica*, he is faced with a considerable difficulty. The ovum of *sylvatica* is not nearly so amenable to the operative techniques as is the ovum of *pipiens*. There are differences in the structure of the surface of the cells that lead to the formation of large exovates in *sylvatica*. The tiny exovate that is produced when the spindle is removed usually continues to enlarge and after a few hours it may have a diameter one-half of that of the remainder of the ovum. Nearly all of the operations where a *sylvatica* ovum is the host are failures because of this excessive growth of the exovate.

In the 1956 operative season the following experiment was attempted. First a nucleus from a *sylvatica* blastula was put into an enucleated *pipiens* ovum. When this embryo reached the blastula stage, an attempt was made to transfer some of its nuclei back into an enucleated *sylvatica* ovum. A total of 43 operations were made and these were the restults:

> One embryo developed normally as far as a late blastula stage; 3 formed partial blastulae; and 39 either did not cleave or cleavage was abnormal.

In the 1957 operative season it was decided to try the reciprocal experiment. First a nucleus from a *pipiens* blastula was put into an enucleated *sylvatica* ovum. When this reached the blastula stage, nuclei were put back into enucleated *pipiens* cytoplasm. Of course, one still has the problem of using the

sylvatica ovum as a host. Nevertheless, one can take advantage of the few blastulae that are obtained and use these as donors. A slight modification of the technique was devised near the end of the operative season and this contributed to the success of the experiments. It was found that the exovates did not enlarge to the usual degree if the enucleations of the *sylvatica* ova were performed in 2 per cent sucrose in 0.1 Holtfreter solution.

In one experiment 3 partial blastulae were obtained from the injection of *pipiens* nuclei into *sylvatica* ova. In the most normal one nearly all of the blastula was cellular. Its nuclei were used in 15 operations with these results:

> Four developed as far as early gastrulae. The amount of invagination was slight but in one a tiny sucker was induced. 3 formed nearly entire blastulae but no dorsal lip developed; 5 formed partial blastulae; and 3 did not cleave.

Nuclei from the other two partial blastulae were used in 52 operations with these results:

> Five formed complete or almost complete blastulae; 9 formed partial blastulae; and 38 did not cleave.

These results must be regarded as preliminary until more can be obtained. Nevertheless it is my feeling that the best embryos obtained show the full abilities of these nuclei. That is, if a *pipiens* nucleus is placed in *sylvatica* cytoplasm where it replicates chromosomes from the *sylvatica* substrates, it becomes modified. The modified *pipiens* nucleus is no longer able to act as a genetically intact *pipiens* nucleus and, when returned to *pipiens* cytoplasm, it is unable to carry the embryo beyond an early gastrula stage.

DISCUSSION

The finding that the rate of cleavage for *pip* × *pip*, (*pip*) × *pip*, *pip* × *syl*, and (*pip*) × *syl* embryos is essentially the same is not an unexpected result. It is in keeping with the general body of information [8] showing that, at the time of fertilization, an ovum has an inherent ability to develop normally to the end of the blastula stage. Within rather broad limits the number and type of chromosomes seems to be of little importance. One is left with the impression that the nucleus is under the firm control of the cytoplasm throughout the cleavage and blastula stages.

The experiments on transplanting a diploid *sylvatica* nucleus to an enucleated *pipiens* ovum are the first to be reported that involve two species of frogs. The inability of this combination to develop beyond the late blastula

stage parallels the situation in the (*pip*) *syl* androgenetic hybrids. Two haploid sets of *sylvatica* chromosomes lead to no improvement in developmental capabilities over one haploid set.

The results of the double transfer experiments are of considerable interest. When a diploid *pipiens* nucleus is allowed to develop in *sylvatica* cytoplasm to the late blastula stage and is then transferred back to an enucleated *pipiens* ovum, it is unable to fulfill its original genetic potentialities. The chromosomes seem to have become modified in some manner—perhaps we could think in terms of a massive transduction—and, as a result, the embryo does not develop beyond an early gastrula stage. It is of interest to note that the best embryos obtained resembled somewhat the *pip* × *syl* diploid hybrids.

It is inappropriate to discuss these results in detail until I have more than the 26 cases reported in this paper. These few facts, however, will set the stage for a possible interpretation.

(1) Sze [11] has shown that the uncleaved ova of *pipiens* contain tremendous amounts of DNA in the cytoplasm. In fact there is more than enough stored DNA to provide for all the nuclei of an early gastrula. Gregg and Løvtrup [2] using a different method find large amounts but not as large as those reported by Sze.

(2) Sze [11] and Gregg and Løvtrup [2] report that there is little or no increase in total DNA and free desoxyribosides in *pipiens* until the late blastula stage.

(3) B. C. Moore [4] finds that the late blastula cells of *pip* × *syl* diploid hybrids have about the same amount of DNA in the nuclei as is present in normal *pipiens* controls. Also, the late blastula cells of (*pip*) × *syl* androgenetic hybrids have about the same amount of DNA in the nucleus as is present in (*pip*) × *pip* controls.

These observations, together with those reported in this paper, can be combined in the following provisional hypothesis. We can assume that the *sylvatica* ovum is similar to *pipiens* in containing large amounts of cytoplasmic DNA and that there is little or no increase in total DNA during the cleavage and blastula stages. This DNA would have been formed under the influence of a *sylvatica* nucleus. When a diploid *pipiens* nucleus is injected into an enucleated *sylvatica* ovum it undergoes a series of mitotic divisions to reach a late blastula stage. It is probable that the *pipiens* chromosomes are formed at each new division from the store of *sylvatica* cytoplasmic DNA. The products are neither normal *sylvatica* chromosomes nor normal *pipiens* chromosomes. That the first is so is shown by the fact that the embryo does

not develop in the normal *sylvatica* manner. That the second is so can be tested by transferring the nuclei back to an enucleated *pipiens* ovum. The chromosomes, which were originally *pipiens* chromosomes, are found to have been altered by their sojourn in the *sylvatica* cytoplasm. They are no longer *pipiens* chromosomes in the genetic sense for they cannot produce a normal *pipiens* embryo when placed in *pipiens* cytoplasm.

SUMMARY

1. New data are offered to show that the cleavage rates of of *pip* × *pip*, (*pip*) × *pip*, *pip* × *syl*, and (*pip*) × *syl* embryos are essentially the same.

2. When a diploid *Rana sylvatica* blastula nucleus is transferred to an enucleated *Rana pipiens* ovum the resulting embryo develops only to a late blastula stage and then stops.

3. If a diploid blastula nucleus of *Rana pipiens* is transferred to enucleated *Rana sylvatica* ova this embryo will also develop to the blastula stage. When nuclei are transferred from these blastulae back to an enucleated *pipiens* ovum, the embryos develop only to an early gastrula stage. It is suggested that the original *pipiens* chromosomes are altered when they are in the *sylvatica* cytoplasm and are replicating from *sylvatica*-type substrates (probably DNA and similar compounds).

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SUBCULTURES OF ADULT MAMMALIAN BRAIN CORTEX *IN VITRO*[1]

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Received September 13, 1957

Until recently, cultivation of tissue from the central nervous system has been largely confined to embryonic tissues, as pointed out by Lumsden [19] in a survey of the literature to 1951. Growth of embryonic nervous tissue is, to a great extent, limited to the outgrowth of neuritic processes from the neurons in the original explant. The perikarya of embryonic neurons generally do not migrate out from the explant, nor do they divide, except under special conditions reported by Levi and Meyer [18]. Hogue [15], however, was able to obtain migrating neurons from gray cortex of human fetuses. Murray and Stout [20] successfully cultivated sympathetic ganglia from adult humans. They described migration of ganglion cells and occasional mitosis in these cells. Costero and Pomerat [4] and Hogue [16], using tissues from adult human cerebral and cerebellar cortex, were able to maintain adult neurons alive for several weeks to several months. No subcultures of adult brain tissue were described.

Geiger and Behar [11] obtained subcultures up to the 6th passage from the precentral area of the adult rabbit cortex, consisting almost entirely of neurons, and maintained them for over six months by using cortisone in the feeding solution and omitting embryonic extract.

In the present paper, brain tissue cultures and subcultures from the occipital, frontal, precentral, temporal, and parietal lobes from the cerebral cortex of adult humans, monkeys, and rabbits were studied, as well as cells from the cerebellar cortex. Special attention was given through successive subcultures to the maintenance of specific characteristics of neurons such as Nissl substance, neurofibrils, etc., over prolonged periods. Regenerative potentialities of neurons, effects of alteration in the glial population, vital staining, and other staining properties, motility, and intracellular activity were studied. Survival time of neurons and mitosis under different conditions, as well as formation of synapses, are described.

[1] This work was done with the aid of a grant from the Mental Health Fund of the Illinois Department of Public Welfare.

TECHNIQUE

Initial cultures were made by planting tissue fragments of approximately 1 mm^3 into a medium consisting of 1 part chicken plasma, 2 parts Tyrode's solution, and 1 drop of chick embryo extract, enough for clot formation, in a 3.5 cm Carrel flask. (Sterile technique was maintained throughout.) When mixed cultures of brain cells were desired, the supernatant fluid, which was added after 24 hours, consisted of 6 parts of homologous serum and 4 parts of Tyrode's solution. To obtain relatively pure cultures of neurons, 0.05-0.01 mg/ml cortisone acetate was added to the supernatant. The supernatant was renewed every 72 hours.

It was possible to make subcultures at any time after 2 weeks. Subcultures were made into Carrel flasks and Maximow slides. In the Carrel flasks cultures were maintained over extended periods of time; they were the source of material for subcultures. The Maximow lying drop slides were used for detailed observation of the outgrowth, and for time-lapse microphotography. Lying drop cultures were also fixed and stained with hematoxylin and eosin, Jacobson's stain, Bodian's (Protargol and gold chloride), Fletcher's thionine stain, and Klüver's stain (Luxol blue and neutral red).

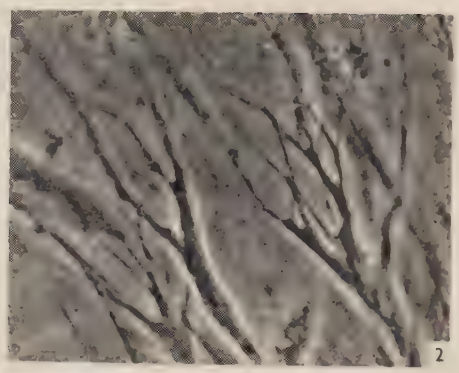
Part of the original explant has usually been transferred into the subculture along with some of the so-called "growth" zone. Recently, however, growth zones without the admixture of original explant have been successfully subcultured from 3rd, 4th, and 5th passage cultures. The maximum life span of neurons in these subcultures has not yet been determined, as they were lost at later stages through technical accidents, such as contamination, incubator defect, etc. Six passages of subcultures, however, have thus far been obtained with an aggregate life span of 15 months. Subcultures of 2nd, 3rd, and 4th passages have each survived from 8 to 13 months. In the 5th and 6th subculture, the life span of the neurons became progressively shorter, and was finally reduced in the 6th passage to 10–15 days, in the serum medium. Cortisone in the supernatant prolonged the life span of the neurons in the 6th passage to 3–4 weeks. From the 4th passage on, the life span of subcultures was significantly prolonged by a Tyrode's extract of brain tissue [13]. In this medium, the 6th passage neurons survived as long as 20 weeks thus far. The use of brain extract in the earliest passages (1st to 3rd) was not practical, as it increased the mesenchymal elements in the outgrowth. Embryonic extract has a similar effect. When brain extract was added, cortisone was omitted from the media [12].

RESULTS

Morphology of neurons in culture.—A variety of neurons in the cultures and subcultures were identifiable as pyramidal cells, giant pyramidal cells, giant polymorphous neurons and bipolar spindle neurons. The cell bodies of the neurons varied in length from approximately 15 to 200 μ or more

Figs. 1–5.—Neurons from 3rd passage subcultures adult cerebral cortex, 4–10 months in culture. Living, phase contrast, × 120. *Figs. 1, 2*, human frontal area; *Fig. 3*, human occipital area; *Fig. 4*, human temporal area; *Fig. 5*, rabbit precentral area, vitally stained neutral red; *Fig. 5a*, area of dendrite from Fig. 5, × 750.

1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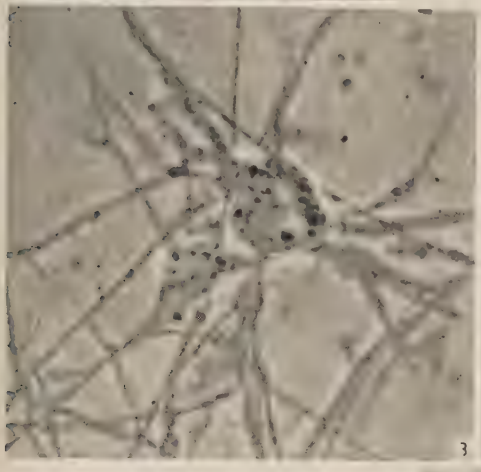
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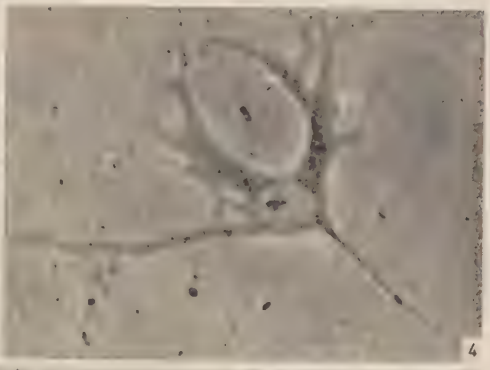
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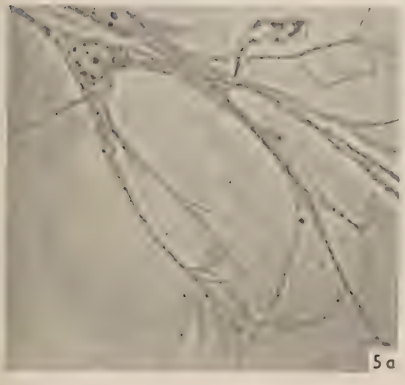
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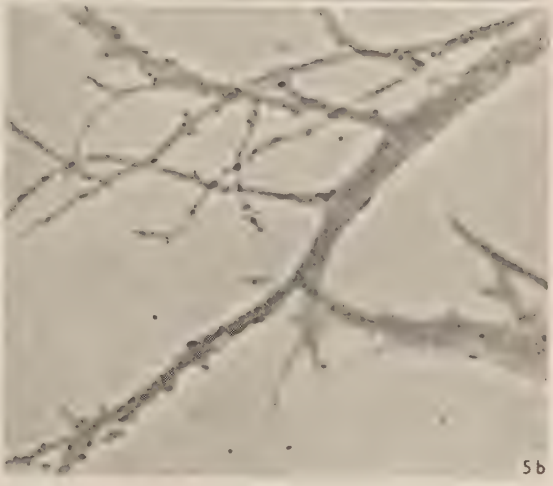
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(Figs. 1–8). Binucleated and polynucleated neurons were seen with all types of media used, but more frequently in cultures containing cortisone. Each nucleus usually contained one large nucleolus. Two or even three may occur, however, particularly in cortisone-containing media. One or two nucleolar satellites are frequently seen. The nucleus may occupy a central or a lateral position and appear round, oval, or streaming, depending on the position of the neuron in relationship to the clot and coverglass, and on the physiological state of the cell. Viewed with phase contrast, the nucleoplasm appears clear. The nucleolus is most often round and of uniform density, but may exhibit a variety of shapes and several areas of differing optical density.

The cytoplasm is granular; and when neurons are thinly spread out rod-like mitochondria which stain vitally with Janus green can be distinguished from other granules, some of which stain preferentially with neutral red. Granules staining with neutral red were also observed in the dendrites, but not in the axon (Figs. 6 and 7). Mitochondria, however, were present in all neurites including the axon (Fig. 8).

Occasionally, neurofibrils could be identified in living neurons. Weiss and Wang [24] described neurofibrils in living bipolar neurons in ganglion explants of chick embryos. The neurofibrils in the living cells should not be confused with aligned mitochondria which is a common occurrence.

In cultures fixed and stained with osmic acid, osmophilic granules could be observed in the cytoplasm of the neurons, but not in the axon hillock. These granules varied in size and osmophilia, depending on the physiological state of the cell. The mitochondria, although weakly osmophilic, could be clearly distinguished. In some cases, neurofibrils were discernible (Figs. 9–10).

In fixed preparations of whole mounts of Maximow lying drop cultures stained with Bodian's protargol and gold chloride [3], it was possible to demonstrate neurofibrils in the cell bodies, and in the processes of neurons

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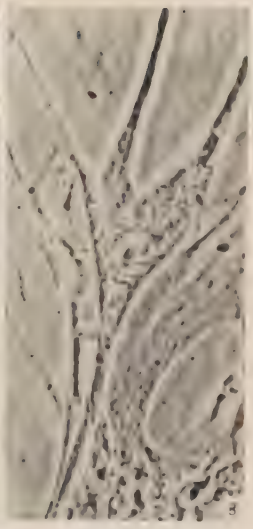
Fig. 6.—Neuron from 3rd passage subculture, human cerebellar cortex. Living, phase contrast, × 200.

Fig. 7.—Neuron vitally stained with neutral red (1:30,000). Note absence of granules axon in hillock and axon descending downward in center of field. 4th subculture, 8 months in culture. Living, phase contrast, × 150.

Fig. 8.—Neuron vitally stained Janus green (1:150,000), after 30 minutes. Note mitochondria in all processes. Living, plain light, × 120.

Fig. 9.—Fifth passage subculture human frontal area stained with osmic acid vapors (2 per cent sol.) for 30 minutes. Note myelinated axons. Plain light, × 75.



throughout successive subcultures (Figs. 11–14). Nissl substance was demonstrated with Fletcher's thionine stain [5] in the cell body of most neurons and in the dendrites, either as particulates, strands, granules, or in vacuoles during the initial stage of reversible vacuolization. The patterns varied with different types of neurons and within the same type, depending on the physiological state of the cell (Figs. 15–18). Whole mount Maximow lying drop cultures were immersed in Fletcher's thionine stain at pH 3.6 for five minutes and subsequently decolorized so that only the Nissl substance or nucleolar material retained the stain.

Jacobson's stain (May–Grünwald–Giemsa) showed the nucleoplasm red and the nucleolus blue, indicating the presence of DNA and RNA respectively. A considerably larger amount of granules stained blue (RNA-containing substance) in the cytoplasm, with Jacobson's stain, than with Fletcher's thionine stain for Nissl substance. This fact, as well as control experiments with ribonuclease digestion indicated that only part of the cytoplasmic RNA is in the Nissl substance. A band of blue appeared around the nucleus. This band varied in dimension, depending on the physiological state of the neurons. In the cytoplasm, sometimes blue or red inclusions were observed with Jacobson's stain (Fig. 19). Partially myelinated axons were demonstrated in mixed subcultures with osmic acid or with Luxol blue counterstained with neutral red. With the latter stain, dendrites were distinguished by the absence of the blue stain present in axons and the presence of neutral red-staining granules (Figs. 20, 21).

Mitosis of neurons.—Occasional mitosis of neurons was seen in fixed and stained preparations of cultures, as well as with time-lapse phase contrast photography in living cultures. These were encountered most frequently in the 1st passage subcultures with cortisone in the media. They were not sufficient in number to sustain a culture. The "growth" of neurons in cultures, even up to the 6th passage, consisted primarily of neurons migrating out from the transferred portion of explant. During mitosis, neurons preserved the characteristic shape of their cell bodies up to late anaphase. Bodian preparations showed neurofibrils to be retained in the processes and in the cell body but they diminished in number and became somewhat coarser and more irregular (Figs. 22–25).

Intercellular granules.—Granules which stain with vital stains were often seen in the clot. Part of this material is undoubtedly derived from disintegrat-

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Fig. 10.—Fourth passage subculture. Osmic acid vapor (2 per cent sol.), 30 minutes. × 300. *Fig. 10a*, area of dendrite, × 300; *Fig. 10b*, glia, same culture, × 300.

10 a

10 b

10 c

ing cells, as described by Hogue [14]. However, cells have been observed to contract and extrude neutral red-staining inclusions into the surrounding medium. The problem of the secreting properties of neurons and glia in culture has hardly been touched.

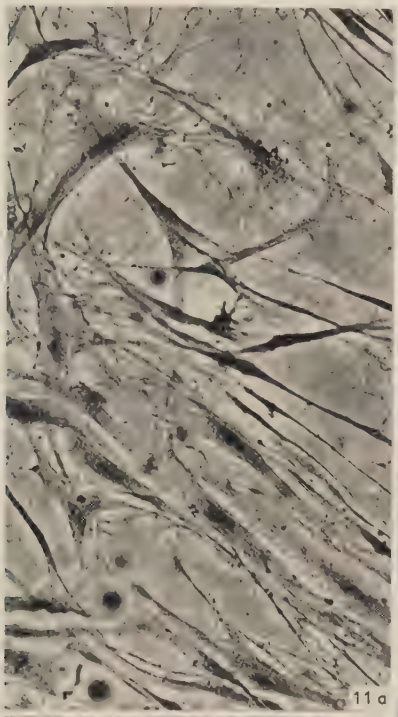
Intracellular activity.—Continuous intracellular movements can be demonstrated in the neurons, using time-lapse microphotography at 4 frames per minute, 160 × with phase contrast. Streaming of cytoplasm throughout the perikaryon and processes, as well as independent movement of cytoplasmic granules to and from the nuclear membrane, or to other areas of the cell, such as actively growing processes, can be seen. Slow pulsatile movements of the cytoplasm of the perikaryon and neurites, particularly of the axon, have been recorded.

Gradual uptake of vital stains was observed by time-lapse photography. The cultures were incubated with vital stains dissolved in Tyrode's solution (Neutral red 1:30,000; Janus Green, 1:150,000 -1:200,000; Methylene blue, 1:150,000 -1:200,000) for 5 minutes, and the excess dye was rapidly washed off with Tyrode's solution. Some dye was retained in the clot, which was then gradually taken up by the cells. Only one of the vital stains, neutral red, proved non-toxic to the neurons over a prolonged period. The observation of Murray and Stout [20] that glial processes do not have neutral red-staining granules was confirmed (Figs. 26–32).

Janus green-stained mitochondria could be observed clearly, but after a while they tended to clump together. Neurons treated with Janus green degenerated completely within a few hours.

Methylene blue was taken up first by granules and particulates in the perikaryon and in the dendrites (Figs. 33, 34). During the following hours, more granules were stained with methylene blue at a slower rate. The nucleolus followed, then, later, the whole cytoplasm became stained. When the whole cytoplasm became stained by the methylene blue, the neurons were already moribund. At this stage, a large number of processes showed rapid bubbling, retraction, and uneven surfaces, with globules. The observations of Costero and Pomerat [4] on the different rates and intensities of methylene blue uptake by individual neurons was confirmed. Neurons cultivated with cortisone usually took up vital stains more quickly than those cultivated without cortisone in the medium.

Fig. 11 *a, b, c, d, e.*—Maximow lying drop whole mounts. Third subcultures, 5 months in culture, from precentral area rabbit cortex. *Fig. 11a, b,* cells at edge of culture; *Fig. 11c, d, e,* cells in body of culture. Stained with Bodian's protargol and gold chloride.



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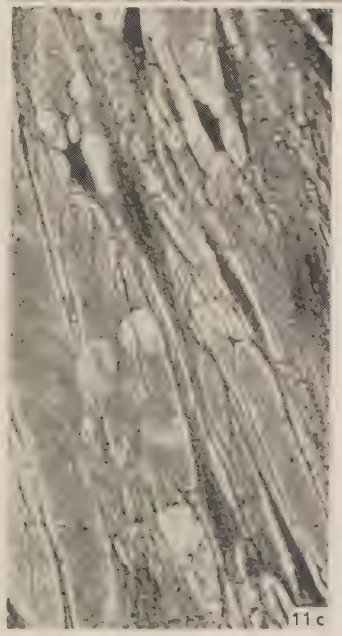

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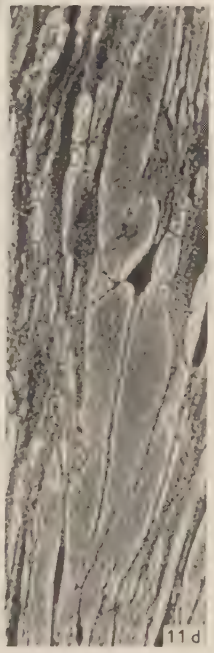

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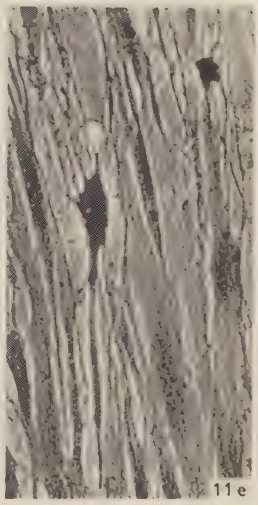

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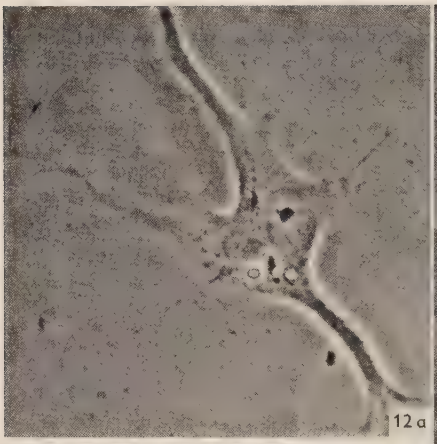
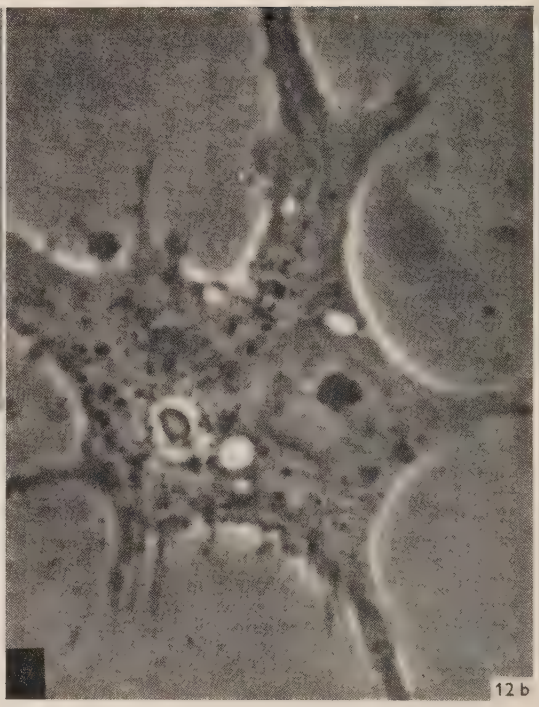




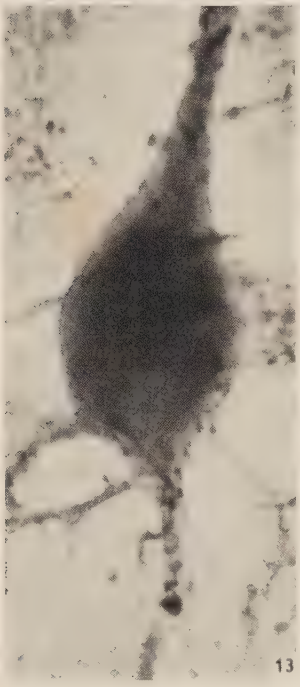
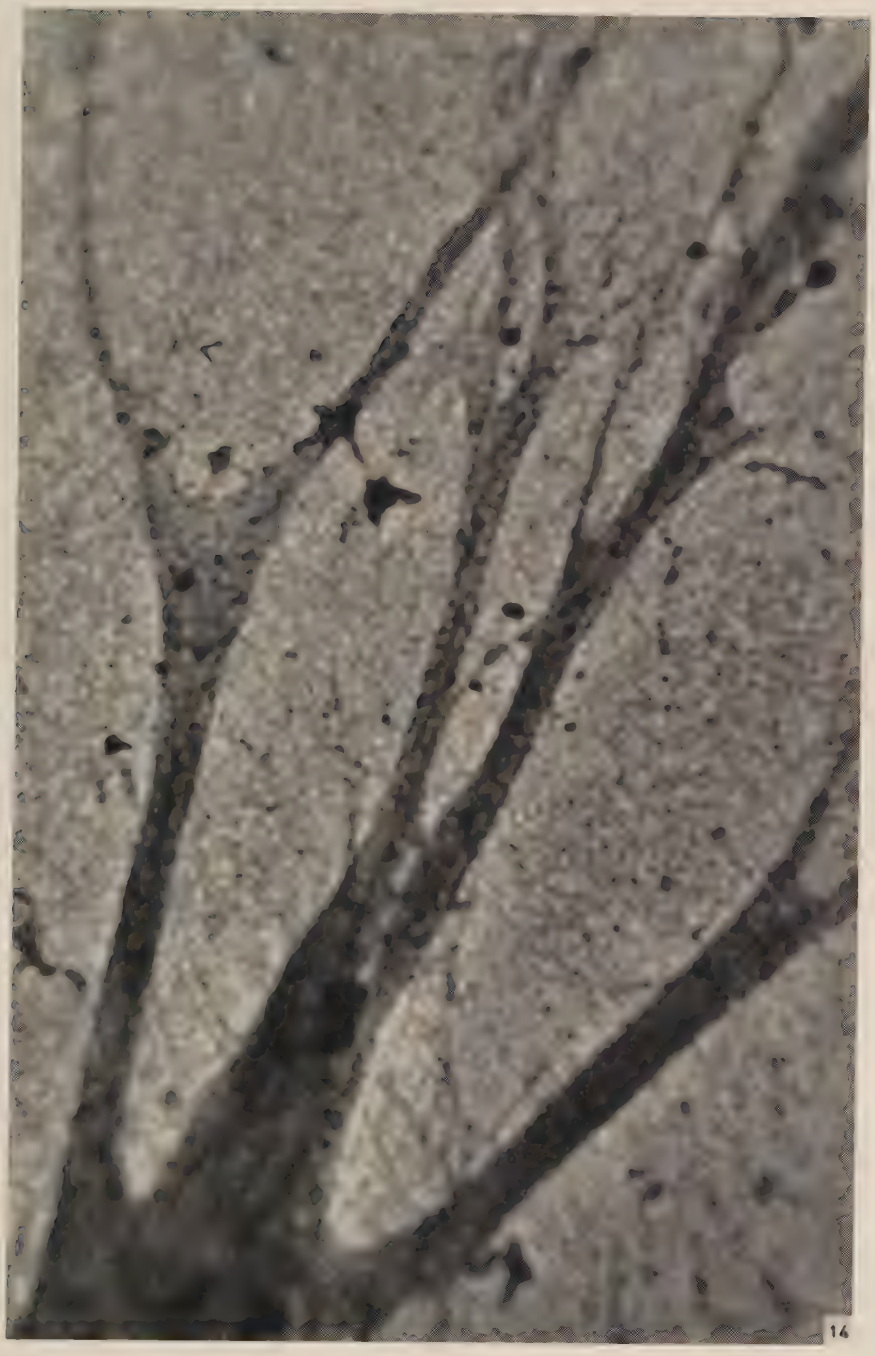


Fig. 12 *a*, *b*.—Neuron from 3rd passage, human subtemporal area, showing neurofibrils in living cell. Phase contrast, × 130 and × 300, respectively.

Fig. 13.—Neuron from whole mount Maximow lying drop subculture, 3rd passage human subtemporal area. Note neurofibrils at less dense area of cytoplasm. Bodian's protargol and gold chloride, × 800.

Fig. 14.—Whole mount Maximow lying drop subculture showing neurofibrils in neurites. Bodian's protargol and gold chloride, × 850, blown up.


14

Activity observed in the nucleus.—A streaming of the nucleoplasm, and, at times, a pinching-off of nucleoplasm into the cytoplasm could be observed [16]. Rolling movements of the nucleus were seen, and also shifting of the position of the nucleus. The nucleus may also change in size and optical density. The nucleolus is round, inert, and of uniform optical density but it may be spread out, change its shape, move toward the nuclear membrane and its optical density vary in different areas.

Transfer of nucleolar material to the cytoplasm was followed in time-lapse sequences with phase contrast microphotography with and without vital dyes. Figs. 39-41, taken from a sequence vitally stained with methylene blue, clearly show the transfer of nucleolar substance into the cytoplasm. This phenomenon was often seen. These observations are in agreement with Hyden's [17] hypothesis that the nucleolus participates in the elaboration of Nissl substance for the cytoplasm. Nucleolar satellites described by Barr and Bertram [2] were observed to move to and from the nucleolus and the nuclear membrane.

Movement of neurons.—The movements of the neuron as a whole in the media are very slow and rarely perceptible over a period of eight hours or more. Neurites may degenerate or retract and neurons round up. Such rounded forms are relatively immobile and soon die. In marked contrast to the neurons, the glia are quite motile, especially when in the form of gütter cells and gemistocytes, or intermediary stages of these, which occur frequently in mixed cultures. Rhythmic contractile movement of glia, described extensively by Pomerat [21] for oligodendroglia, were also observed.

In relatively pure neuron subcultures, neurons can migrate out from the explant with or without other types of cells being present in the vicinity. This is contrary to the belief of Levi and Meyer [18], who thought that accessory cells were essential for the migration of neurons.

The neurites emerge from the explant first, often exhibiting undulating movements (Fig. 36). These processes usually can be identified as dendrites by the presence of neutral red-staining granules. They are followed by an elongated perikaryon, the emergence of which is accomplished by rapid intracellular movements of cytoplasm toward the neurites and by changes in shape of the perikaryon. Finally the axon emerges. After migrating away

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Figs. 15-18.—Neurons stained for Nissl substance. Fletcher's thionine stain. *Fig. 15a, b*, fourth subculture, human occipital area, 6 months in culture, ×98; *Fig. 16*, human parietal area, 4th subculture, × 390; *Fig. 17*, human temporal area, 4th subculture, × 390; *Fig. 18*, rabbit precentral area, 4th subculture, × 390.

15a

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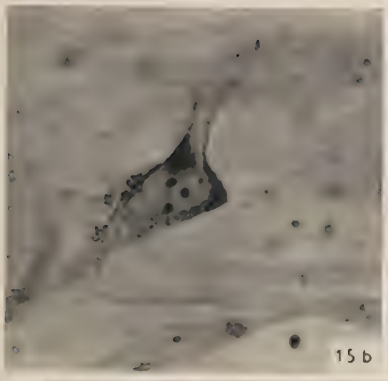

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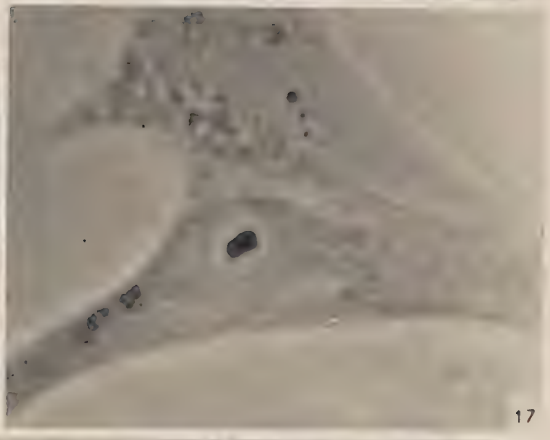

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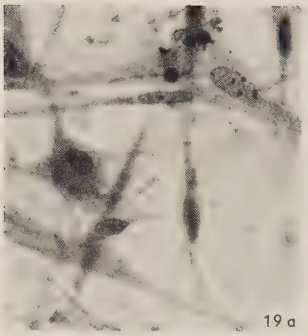
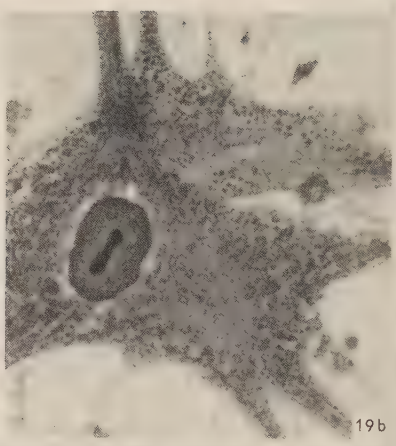




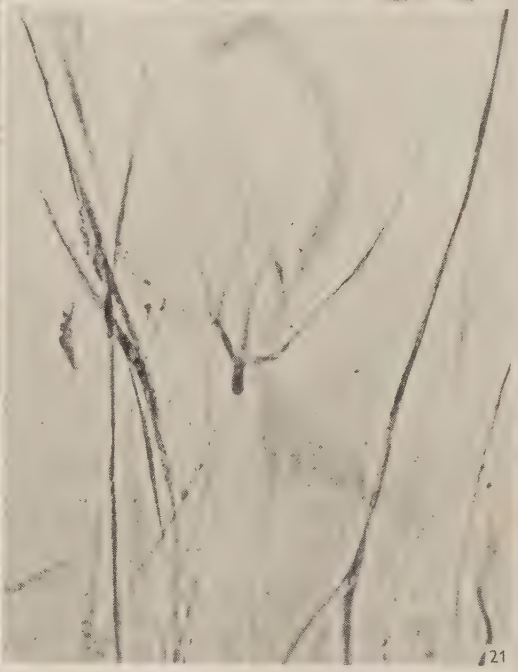
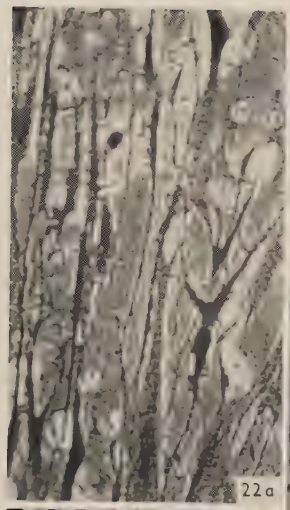


Fig. 19*a*, *b*.—Neurons from rabbit precentral area, 5th subculture, Jacobson's stain. *Fig. 19a* shows different distribution and heavier staining of neurons than glia or mesenchyme cells. × 110. *Fig. 19b*, neuron. × 275.

Figs. 20, 21.—Neurites stained with Klüver's Luxol blue and neutral red. Note darker staining myelinated areas of axon often associated with oligodendroglia and neutral red-staining granules in dendrites.

Figs. 22–25.—Neurons in telophase from 1st subculture, precentral area, Bodian's protargol and gold chloride. *Fig. 22a*, × 75; *Fig. 22b*, same as 22*a*, showing coarse neurofibrils, × 750; *Fig. 23*, × 75; *Fig. 24*, × 300; *Fig. 25*, × 750.



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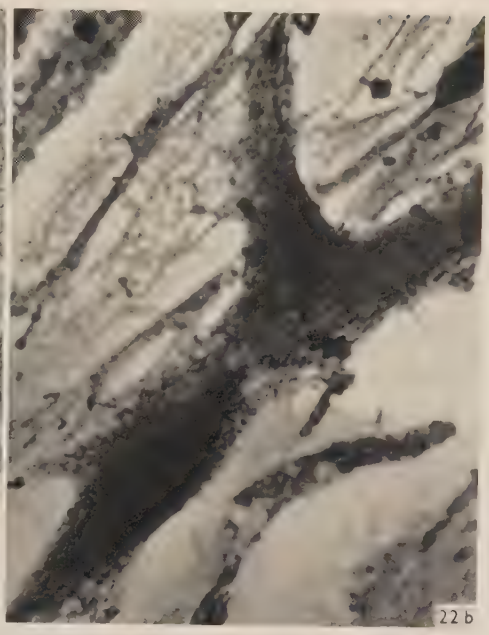

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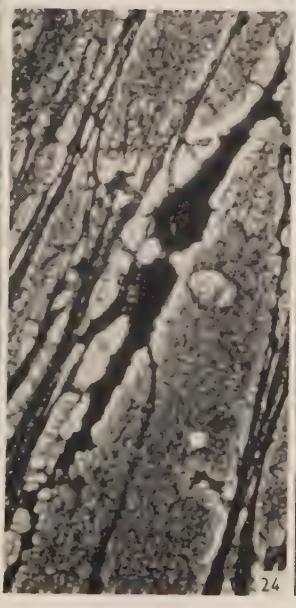

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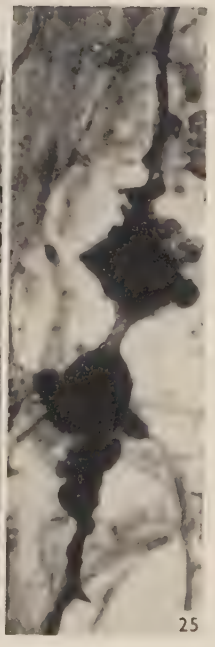

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from the explant, the perikaryon gradually assumes its characteristic shape and size, which may vary, depending upon its relation to the clot or glass surface. The neurites may grow in length and put out more collaterals, or, on occasion, they may retract or flatten out. Speidel [22], in studying the growth of nerve fibers in the tadpole tail, reported similar observations.

Formation of synapses.—In the course of this work, numerous instances of a definite orientation of the processes of one neuron toward the cell body or processes of another neuron have been observed. Such contact may be transitory, lasting only for a few hours, or the contacts once established may persist for days, and, in a few observations, for several weeks. These points of association or synapses, when stained with Bodian's protargol gold chloride stain, usually are seen either in the form of cytoplasmic bridges similar to those described by Young [25] in cephalopods, or in a form often seen in histological preparations of cortex. These latter endings show a thickening toward the end of the process, from which numerous fine branches and end feet (boutons terminaux) emerge (Figs. 42, 43).

When such synapses are observed with time-lapse and phase contrast photography, the thicker endings of the neurites show amoeboid-like movements. The fine fibers and end feet also show movement. They may retract or swell. The mitochondria and other granules in the end feet are extremely motile at the area of contact. The region of contact of the associated cell shows a very clearly circumscribed area in which numerous granules move very rapidly to and fro (Figs. 44–49). The increased motility at points of contact is persistent.

Dendrites of multiple dendritic cortical neurons establish enduring association with dendrites of other similar neurons (Fig. 10). Processes of neurons may also associate with glia and vice versa (Figs. 37, 38).

Nissl substance.—Certain granules or particulates visible with phase contrast microscopy in the perikaryon and dendrites of neurons in culture are, in all likelihood, liponucleoprotein aggregates identifiable with Nissl substance when compared with fixed and stained thionine preparations. On vital staining with weak solutions of methylene blue (1:150,000–1:200,000 conc.) these granules, present in the perikarya and dendrites, are the first to con-

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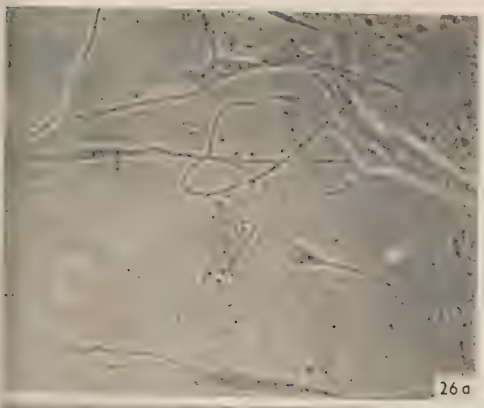
Fig. 26, 26*a*, *b*, *c*.—From 3rd passage precentral area, living, phase contrast. *Fig. 26 a*, general area, × 110; *Fig. 26 b*, Same culture, glia and mesenchyme vitally stained with neutral red, × 280; *Fig. 26 c*, *d*, same culture, portions of two neurons vitally stained with neutral red, × 280.

Fig. 27*a*, *b*.—From 3rd passage subculture, human parietal area, vitally stained neutral red. × 280. *Fig. 27 a*, note absence of neutral red-staining granules in processes of glia; *Fig. 27 b*, tips of dendrites, same culture; note neutral red granules.



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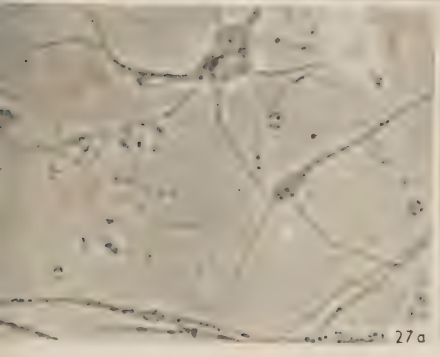

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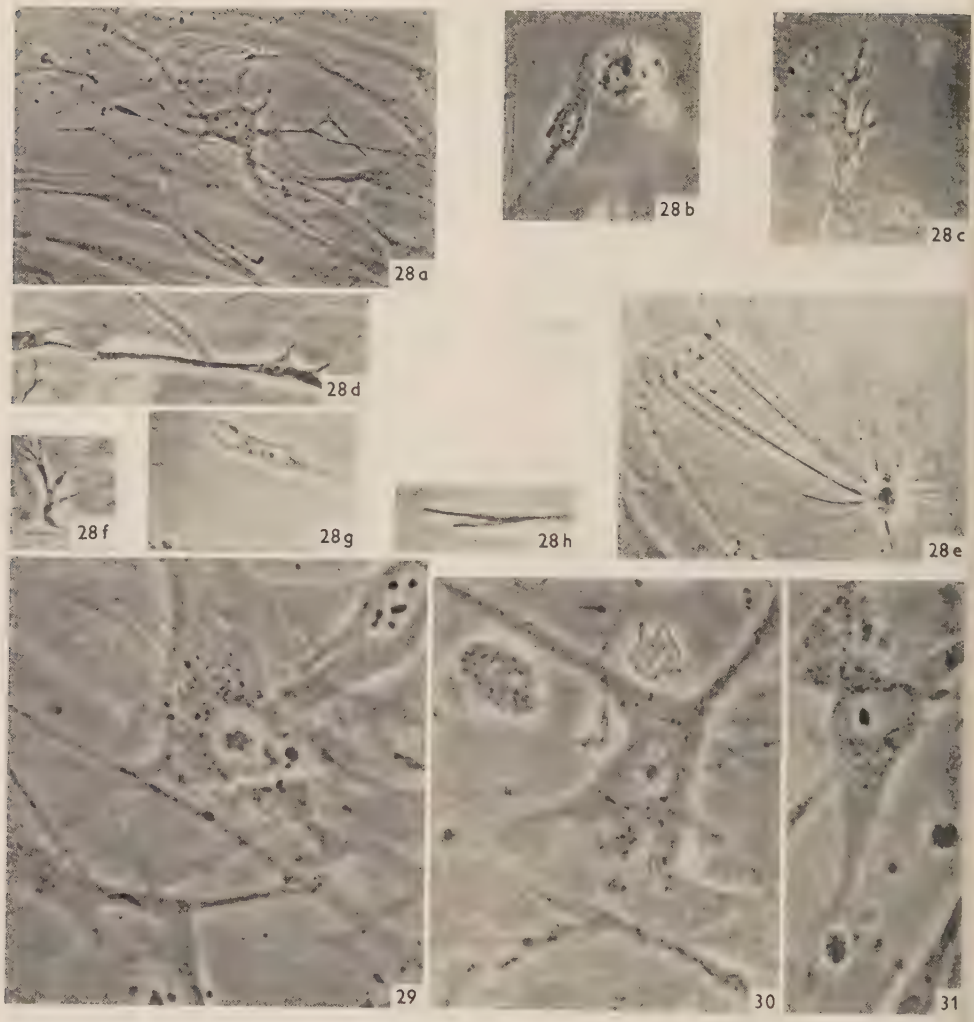
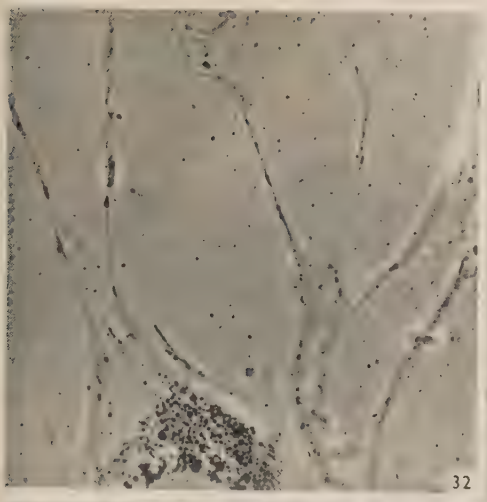
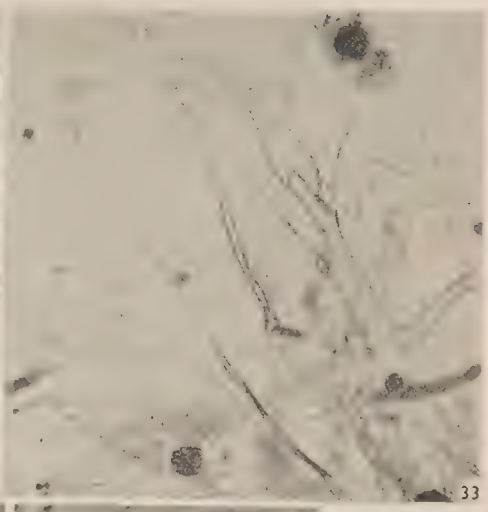
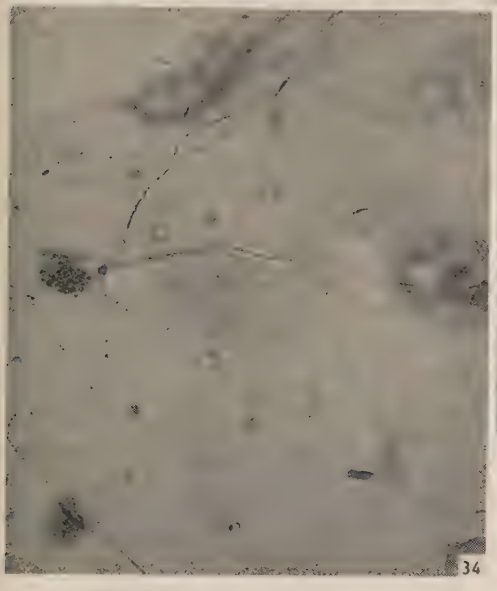


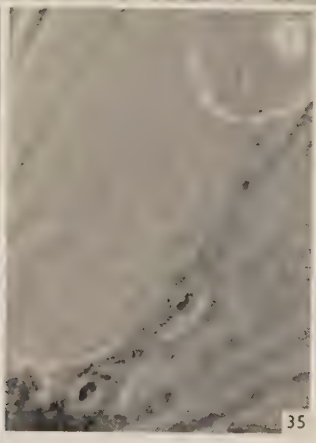
Figs. 28 *a–g*.—Second passage culture, human subcortical white matter, living. Phase contrast, × 150. *Fig. 28 a* shows general field with glia and thinning spread out mesenchyme cells; *Fig. 28 b g* show groups of glia and individual glia cells.

Figs. 29–31.—Subcultures, temporal area human cerebral cortex. *Fig. 29*, unstained, phase contrast; note dense granules in neuron absent in glia and underlying mesenchyme; × 120; *Fig. 30*, same type neuron vitally stained neutral red, × 120; *Fig. 31*, same type neuron Fletcher's thionine stain, × 300.









Figs. 32–35.—Third passage subcultures from rabbit cerebellar cortex. *Fig. 32*, vitally stained neutral red in cell body and in dendrites, × 280; *Figs. 33–34*, vitally stained methylene blue (1:200,000) after show ng granules in dendrites and cell body absence in axon, × 110; *Fig. 35*, Fletcher's thionine stain showing Nissl granules in dendrites and cell body, × 280.

centrate the dye; they also stain vitally with neutral red. Viewed with time-lapse phase contrast microscopy they may be seen to move to and from the nuclear membrane and to active areas such as sites of dendritic formation. They may be present in aggregates or small granules, and their shape and size may vary during a time-lapse sequence of several hours. They are absent from the axon hillock and axon (Figs. 29–35).

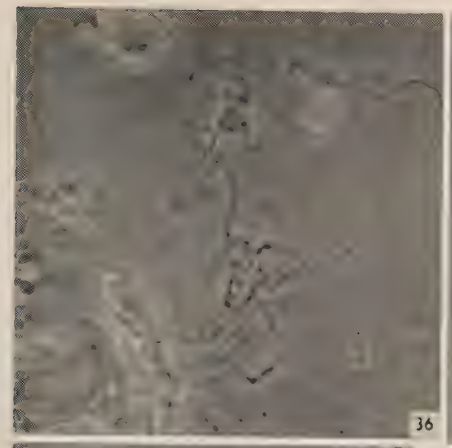
Vacuolization occurring in neurons may cause a marked reduction in the quantity of Nissl substance. This phenomenon may occur spontaneously, but was most often observed in thinly spread-out neurons and was accelerated by light, methylene blue, and drugs. The formation of vacuoles in neurons has been followed with time-lapse photography. The vacuoles usually appear first in the perinuclear region, and spread gradually throughout the cytoplasm. Finally, vacuoles have been observed to appear in the nucleus. If vacuolization has not proceeded to an advanced stage, it is reversible, by washing, feeding, and returning the culture to the incubator. Beyond a certain point, vacuolization is irreversible. Within 12 to 15 hours, the smaller vacuoles coalesce into several large vacuoles, the neuritic processes retract considerably, and degenerated (Fig. 50). Finally, the whole perikaryon degenerates. During vacuolization, some granules gradually disappeared and other concentrated around the vacuoles. When the vacuolization was reversed, the granules, on reappearance, were smaller and more diffusely dispersed throughout the cytoplasm. It often required 48 hours or more until they resumed their original appearance. When vacuolization occurs in neurons which are being vitally stained with dilute concentrations of methylene blue, as described above (1:150,000–1:200,000), the early methylene blue-staining granules (Nissl substance) gradually disappear from the cytoplasm within one hour or less. The vacuoles are first a bright blue color and become colorless within 10–20 minutes. Figs. 51 and 52, taken from a time-lapse sequence of three hours, illustrate this.

Fig. 36.—Third subculture human parietal area neurites and neurons and gütter cells seen emerging from explant. From time-lapse movie, phase contrast, × 130.

Fig. 37.—Different types of glia associated with neuron. 2 per cent osmic acid vapor, one hour, phase contrast. × 320.

Fig. 38.—Glia in lower right corner associated with neuronal dendrite. Note unstained cytoplasm of glia, in contrast to neuron. Fletcher's thionine stain. Phase contrast, × 320.

Figs. 39, 40, 41.—Reproductions of single frames at intervals of half hours from a time-lapse movie. Vitally stained methylene blue (1:150,000). Living, phase contrast, × 130.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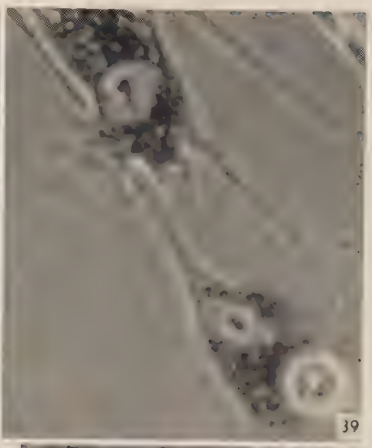
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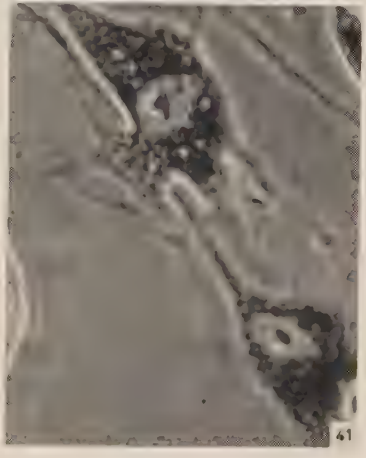
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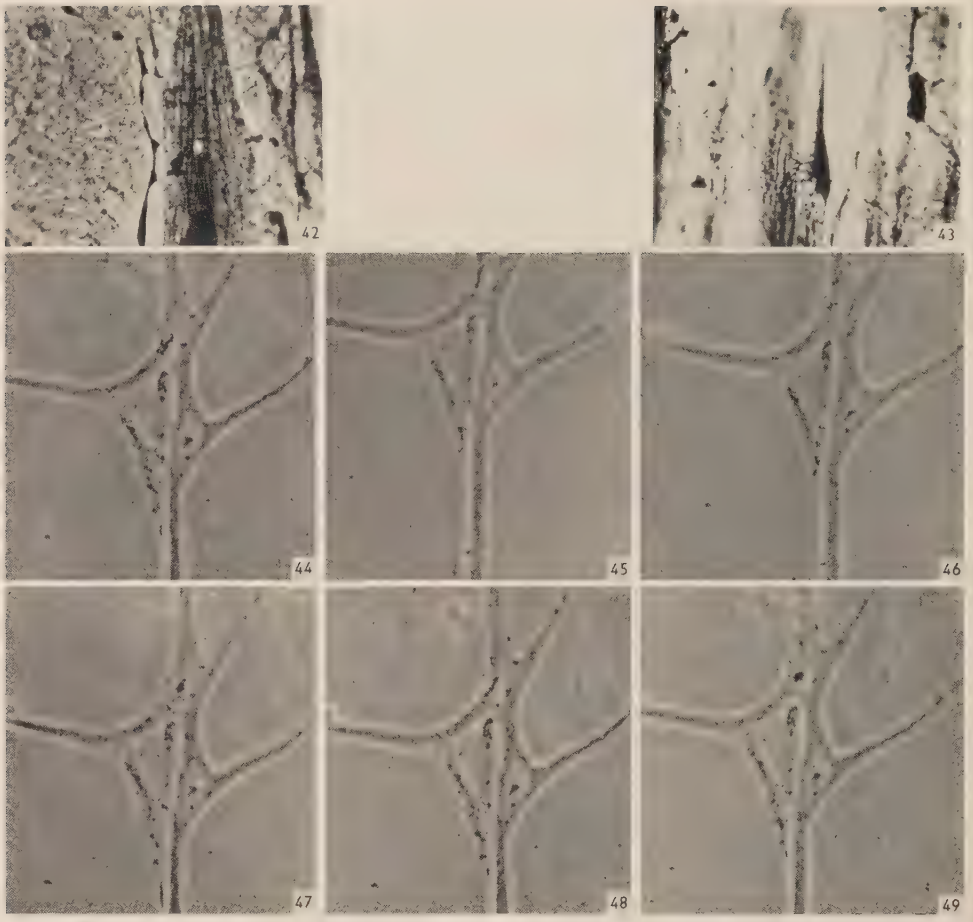
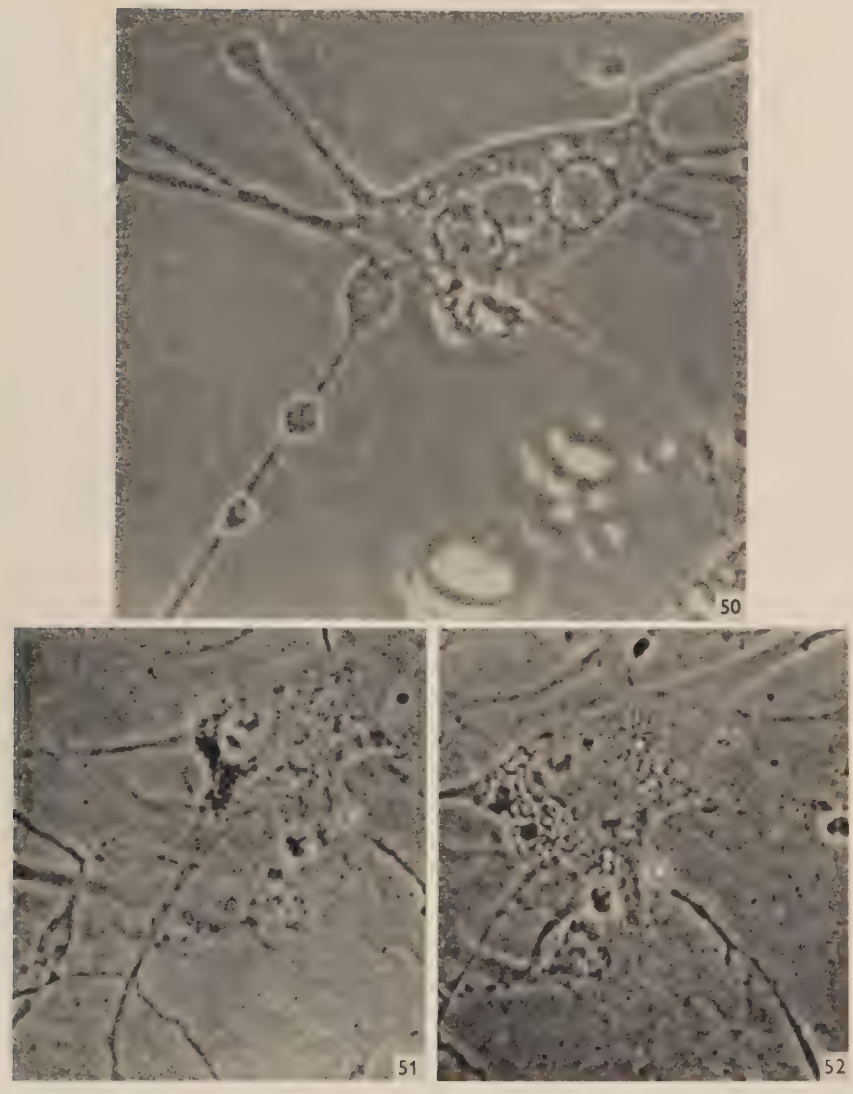


Figs. 42, 43.—Synapses from 4th subculture, rabbit precentral area. Bodian's protargol and gold chloride. × 650.

Figs. 44–49.—Reproductions from single frames of time-lapse movie taken at half hour intervals, showing changes at area of synapse. Living, phase contrast, × 100.

DISCUSSION AND CONCLUSIONS

It has been demonstrated in the foregoing that neurons from adult mammalian brain cortex can be subcultured and maintained for over a year *in vitro*. Hitherto, neurons have not been maintained for such a length of time, nor have subcultures been reported. The success in the present work is, in the author's opinion, due to the type of technique used, the omission of embryonic



Figs. 50–52.—Vacuolization of neurons. *Fig. 50*, degenerating neuron vitally stained neutral red; living, phase contrast, × 160; *Figs. 51–52*, neuron vitally stained with methylene blue (1:150,000) from single frames of time-lapse movie, taken 2 hours apart; living, phase contrast, × 160.

extract from the feeding solution, the judicious use of cortisone, the incorporation of a brain extract as described, and last, but not least, the omission of large concentrations of antibiotics. In the course of this work, it was observed that concentrations of penicillin larger than one hundred units per ml and

50 units of streptomycin per ml noticeably reduce the survival time of neurons. Even with these amounts, survival time was shorter than without antibiotics. This sensitivity of neurons to antibiotics is in sharp contrast to the relative insensity of fibroblasts, epithelium, and muscle cells. The neurons are also distinguished by their sensitivity to exposure to intermediate temperatures between 15°C and 34°C for periods of a few hours. Such exposure seldom damages other cells.

The neurons, glia, and other cells in these cultures have been identified by their characteristic shapes, size, and other morphological properties in the living state, by phase contrast time-lapse microscopy, by vital staining, and staining properties with a number of stains in fixed preparations. In each case, the convergence of these criteria was used for identification, and no conclusion was based on any single property. These neurons retained special staining properties also for Nissl substance and neurofibrils, and gave evidence of myelinization through six successive subcultures. Although the neurons as a whole are relatively immobile, continuous intracellular streaming of the cytoplasm and movements of organelles, nucleolus, and nucleus were recorded by time-lapse microphotography. The cytoplasmic flow, as well as pulsatile movements were directed from the perikaryon to the neurites, and back, showing that cellular material elaborated in the perikaryon descends into the neural processes. Similar conclusions were reached by Weiss and Hiscoe [22], who obtained an accumulation of axoplasm in areas above compressed fibers. Elsewhere [9], it has been shown that addition of serotonin (1—5 γ/ml) to the cultures induces a very slow but forceful rhythmic pumping in neurons, actively forcing the cytoplasm down the processes.

Changes in the consistency of the cytoplasm are indicated by the variations in the rates of movement of intracellular elements [7, 8]. Such changes are often seen occurring spontaneously, but can also be influenced by drugs. Rapid movements of small particulates in the cytoplasm of spinal ganglion cells, dissected out in an uninjured state, were recorded by Adamstone and Taylor [1]. Injury to these cells resulted in loss of these movements.

The pattern of distribution and the amounts of Nissl substance may vary at different times in the same type of neuron, or even in the same neuron. Changes in the distribution of Nissl granules were often accompanied and followed by a transfer of nucleolar substance to the cytoplasm, and by changes in size and optical density of the nucleus and nucleolus. These phenomena seem to be interrelated.

Increased rates of transfer of nucleolar material following electrical stimu-

lation, or addition of metrazol, serotonin, and during recovery from NaBarbital has been shown [6–9].

Time-lapse photography has demonstrated that synaptic areas are strikingly dynamic regions and not the fixed structure usually described. These observations, which show circumscribed areas at the points of contact (synapses?) possessing a high rate of motility of granules, small fibers, and end feet, may explain why in fixed preparations not all the boutons terminaux are found in direct contact with the adjacent cell. Elsewhere, it has been shown that the pattern of movements at these synapses in tissue culture may be modified by drugs and by stimulation [7–9]. Speidel [22] has observed the swelling at endings of nerve fibers in the tadpole tail when metrazol was applied.

Mitosis of neurons occurs occasionally in cultures and early subcultures of adult brain cerebral cortex. This is in marked contrast to the absence of mitosis *in vivo*. There are more frequent mitosis of neurons in early subcultures when cortisone is present in the medium. Cortisone may act directly on the neuron, or may, by its action on the glia, cause a change in the environment which permits mitosis in a predisposed neuron. The number of mitoses found in cultures is exceedingly small compared to those found in cultures of other adult tissues growing *in vitro*, and is not generally the source of neurons appearing in subcultures, which result from migration from the explant.

SUMMARY

1. Relatively pure cultures of neurons from adult cerebral cortex (man, monkey, rabbit) have survived through six subcultures for periods of 8 to 13 months. Subcultures have also been obtained from cerebellar cortex.

2. The presence of neurofibrils and Nissl substance in neurons was demonstrated by selective staining and by phase contrast photography.

3. The variability of the Nissl pattern is discussed.

4. Dendrites contain granules which stain with neutral red or methylene blue vitally and in fixed preparations. These are absent in the axon hillock and axon, as well as in glial fibers. Myelinated axons in subcultures have been demonstrated with both Luxol blue and osmic acid.

5. The selective uptake of methylene blue by neurons is discussed.

6. Continuous streaming of cytoplasm throughout the perikaryon, dendrites, and axon, as well as slower movements of granules to and from the nuclear membrane, and other areas of the cell were demonstrated. A very slow

pulsatile activity of cytoplasm in the perikaryon and axon can also be distinguished.

7. Movements of the nucleus, streaming, and changing in its size and density, and breaking off of nuclear substance into the cytoplasm are described.

8. Transfer of nucleolar material to the cytoplasm has been recorded.

9. In contrast to the glia, movement of the neuron as a whole is very slow and may only be seen if observed over a period of days.

10. Neural processes and their branches extend or retract and their endings exhibit an amoeboid type movement.

11. Definite patterns of association or synapses may be established between neurons. The type of movement at areas of synapse is described.

12. Occasionally, mitosis of neurons occurs. This has been seen most often in early subcultures when cortisone is present in the media.

I wish to express my gratitude to Drs. Sugar, Patrick, Manfredi, and Bird, of the Department of Neurology, University of Illinois College of Medicine, for their cooperation in obtaining the human material used in these studies. I also want to thank Richard A. Crosse for assistance in photomicrography, and Hilda B. Sanders for assistance in maintaining tissue cultures.

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STUDIES IN HISTOCHEMISTRY. XLVIII

DRY MASS OF MAST CELLS MEASURED BY INTERFERENCE MICROSCOPY AND X-RAY ABSORPTION[1]

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Received September 14, 1957

The purpose of this study was to measure the dry mass of mast cells from the peritoneal cavity of the rat by two independent methods, interference microscopy and X-ray absorption, and to determine the effect of the histamine liberator, 48/80,[4] on the mass. Interference microscopy is particularly advantageous for measurements on isolated cells surviving in physiological media, while X-ray absorption measurements of mass require dried samples.

Mast cells contain histamine, heparin and serotonin, and the liberation of these compounds *in vivo* is of considerable functional importance. Injection of animals with drugs which act as histamine liberators cause some degranulation and disruption of mast cells [1, 9, 15, 17, 19], and 48/80 has also been reported to have this effect *in vitro* [15, 16]. Histamine liberators are not specific but also release heparin from tissues, apparently from their mast cells [14, 18], as well as serotonin [3, 20]. Accordingly, it might be expected that the loss of these compounds from mast cells would be reflected in a loss of cell mass, and it was to test this possibility that measurements were made with and without 48/80 in cell suspensions used for interference microscopy.

EXPERIMENTAL

Interference Microscopy

Adult male albino rats of the Sprague Dawley strain were used. Mast cells were isolated in a sucrose density gradient from peritoneal washings by the procedure described by Glick *et al.* [11], except that heparin was omitted from the Hanks'

[1] This study was supported by research grants No. H2028 and RG3911 from N.I.H., U.S. Public Health Service, an Institutional Research Grant from American Cancer Society, and by grant from Louis W. and Maud Hill Family Foundation.

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[4] A condensation product of *p*-methoxyphenethylmethylamine and formaldehyde, kindly supplied by Dr. Edwin J. deBeer, Burroughs Wellcome & Co., Tuckahoe, N.Y.

solution to avoid interference with 48/80. In the final preparation cells from each rat were suspended in 1 ml of Hanks' solution.

A strip of Scotch tape was fastened along each long edge of a microscope slide to prevent the cover slip from pressing on the cells, and between the strips a ring of petroleum jelly-paraffin mixture, 1:1 (prepared by heating) was used to prevent evaporation of the cell suspension placed in the center of this ring and sealed with a cover slip. A Dyson interference microscope (Cooke, Troughton & Simms, Ltd.) was used with a mercury vapor lamp and a filter (Ilford 807, max. transmission 5460 Å).

Measurements were made up to 2 hours after isolation of the cells, and little, if any, change in cell morphology or mass occurred during this period. When 48/80 was added to the Hanks' solution no measurements were made before 30 minutes. Mass per unit area, m/A, equals ϕ/X, where ϕ is optical path difference expressed in wavelengths of the green light used, and X is a constant representing the change in refractive index per unit of density of the mass, $X = 100\ \alpha$ (α, the specific refractive increment, is 0.0018 for protein) [2, 6]. The difference in refractive index, $\Delta\nu$, between Hanks' solution and water at 24°C, the experimental temperature, was found to be 0.0016. Considering the cell as a homogeneous sphere with diameter, d, the volume, V, equals $d^3\pi/6$ and the total mass, m_T, would be given by V times the mass per unit volume, $m/A/d$ (measured through the diameter of the sphere, *i.e.* in the center of the circular image of the cell). Then

$$m_T = \frac{m/A}{d}\,\frac{d^3\pi}{6} = \frac{m\,d^2\pi}{A\,6}. \qquad (1)$$

The correction for the difference, $\Delta\nu$, between Hanks' solution and water is $(0.0016/X)$ g/ml, and the correction in the total mass becomes $(0.0016/X)V$ or $(0.0016/X)\ d^3\pi/6$. Accordingly,

$$m_T(\Delta\nu\ \text{corr.}) = \frac{m\,d^2\pi}{A\,6} + \frac{0.0016\,d^3\pi}{X6} = \frac{\pi d^2}{6}\,(m/A + 0.0089\,d). \qquad (2)$$

A microscope with a micrometer ocular was used to measure d.

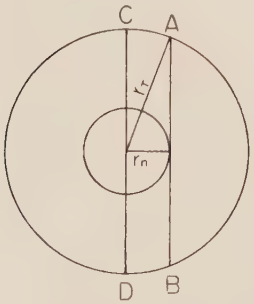

Fig. 1.—Relationship of inner nuclear sphere to outer spherical shell of a mast cell.

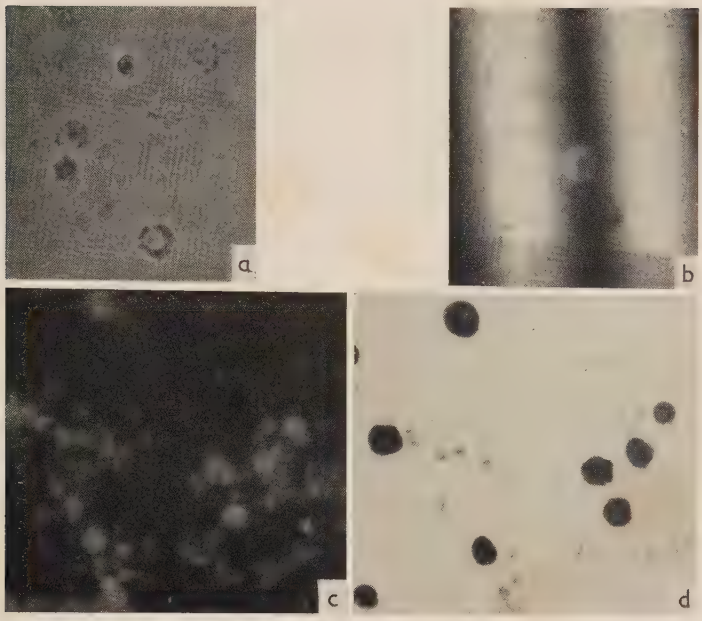

Fig. 2.—(*a*) Phase contrast photomicrograph (× 215) of mast cells in Hanks' solution, isolated from peritoneal washings of a rat by centrifugation in a sucrose density gradient. (*b*) Interference photomicrograph of a cell in (*a*). (*c*) Microradiogram (× 215) of a mast cell imprint from rat omentum. (*d*) Preparation used for (*c*) stained with toluidine blue for identification of mast cells (× 215). Note corresponding cells in (*c*) and (*d*).

Phase contrast and interference microscopy indicate that the mast cell is not homogeneous but appears to consist of an inner nuclear sphere surrounded by a denser spherical shell, Fig. 2 *a*, *b*. If r_n and r_T are the radii of the nuclear region and total cell, respectively, then the distance *AB*, Fig. 1., is $2\sqrt{r_T^2 - r_n^2}$, and the mass per unit volume in the cytoplasm, $(m/V)_c$, through *AB* adjacent to the nuclear zone is

$$(m/V)_c = \frac{(m/A)_c}{2\sqrt{r_T^2 - r_n^2}}. \tag{3}$$

The mass of the cytoplasmic shell, m_c, becomes

$$m_c = \frac{(m/A)_c}{2\sqrt{r_T^2 - r_n^2}} \left(\frac{4\pi r_T^3}{3} - \frac{4\pi r_n^3}{3} \right), \tag{4}$$

and the mass of the nuclear sphere, m_n, is

$$m_n = (m/V)_n \left(\frac{4\pi r_n^3}{3} \right). \tag{5}$$

Micrometer ocular measurements showed r_n to be about 35 per cent of r_T. The value of $(m/A)_{cn}$, where *cn* refers to the nuclear region consisting of nucleus with over- and underlying cytoplasm, was found to be about 80 per cent of that in the cytoplasm, $(m/A)_c$, adjacent to the edge of the nuclear region. Since the values of $(m/A)_{cn}$ and $(m/A)_c$ are measured, the former can be used to obtain $(m/V)_n$ from the following:

$$(m/A)_{cn} = 2\, r_n\, (m/V)_n + 2\, (r_T - r_n)\, (m/V)_c, \tag{6}$$

or

$$(m/V)_n = \frac{(m/A)_{cn} - 2\, (r_T - r_n)\, (m/V)_c}{2\, r_n}. \tag{7}$$

$m_T = (m_c + m_n)$, and when this is calculated using equations 3–7, m_T is about 4 per cent greater than that obtained by eq. (2), when $(m/A)_c$ is used for the value of (m/A) in the latter. The reason that this correction turns out to be small is because the reduced thickness at the point of cytoplasmic measurement (*AB*, Fig. 1) compared to the center (*CD*) is almost entirely compensated by lower nuclear density. Accordingly, the interference data were calculated from the following approximate correction of eq. 2:

$$m_T\,(\text{final corr.}) = \frac{1.04\,\pi d^2}{6}\left[(m/A)_c + 0.0089\, d\right]. \tag{8}$$

The value of m_T in eq. (8) is obtained by using the value of $(m/A)_c$ which is itself uncorrected for $\Delta\nu$. However, in order to compare $(m/A)_c$ with and without 48/80 treatment, it is necessary to obtain the actual values corrected for $\Delta\nu$. This was accomplished by adding the factor $\left[\left(\frac{0.0016}{0.18}\cdot 10^{-12}\right) AB\right]$ to $(m/A)_c$ in each case. Since $AB = 2\sqrt{r_T^2 - r_n^2}$, the correction factor becomes $[(0.0089 \cdot 10^{-12})\, 2\sqrt{r_T^2 - r_n^2}]$ or $1.66 \cdot 10^{-13}$ for the Group I mean. The corrected data are given in Table I.

X-Ray Absorption

The technique of the measurement of mass and the calculations followed essentially those given by Engström and Lindström [7]. The apparatus employed was a Philips CMR5 contact microradiography unit [4], and Lippmann photographic film (Gevaert) was used. The exposures were made for 1 minute with 2 kV and 2 mA. Parlodion was used to make the base foils and the calibrating step wedges. A ratio of 1.37 was calculated for the mass absorption coefficient of Parlodion to that of protein [7, 13], based on the

TABLE I. *Mass of peritoneal mast cells from the rat.*

No. of cells	Mean diam. (μ)	Std. error	Uncorr. (for $\Delta\nu$) mean cytoplasm mass/area (g $10^{-13}/\mu^2$)	Std. error	Corr. mean cytoplasm mass/area (g $10^{-13}/\mu^2$)	Final corr. mean total mass/cell calc. for sphere (g 10^{-10})	Std. error	Conc. 48/80 (μg/ml)
			Untreated cells measured by interference microscopy					
35	19.8	0.3	6.7	0.3	8.3	1.8	0.2	
11	18.5	0.4	7.8	0.6	9.2	1.7	0.3	
21	21.0	0.3	7.5	0.3	9.2	2.2	0.2	
30	20.2	0.2	6.5	0.3	8.2	2.2	0.1	
p I mean cells	19.9	0.1	7.1	0.2	8.8	2.0	0.1	
			Cells treated with 48/80 measured by interference microscopy					
17	21.3	0.4	5.1	0.2	6.9	1.7	0.1	1.0
15	21.4	0.4	5.3	0.2	7.0	1.8	0.2	2.2
23	20.7	0.3	6.1	0.3	7.9	1.9	0.1	2.5
26	21.3	0.5	5.7	0.3	7.5	1.9	0.2	3.0
p II mean cells)	21.2	0.2	5.6	0.1	7.3	1.8	0.1	
icance difference , groups nd II	<0.001		<0.001		<0.001	0.3		
ntreated cells from same rat measured by interference microscopy (IM) and X-ray absorption (X)								
M) 15	19.4	0.3	8.4	0.5	9.8	2.0	0.1	
X) 25[a]	7.6	0.3	75.8	4.8	75.8	2.3	0.1	

[a] Cells dried for measurement.

following elementary analysis of the Parlodion used: C 26.43 per cent, H 2.83 per cent, N 11.90 per cent, O 58.84 per cent.

Adult male albino rats of the Sprague Dawley strain were also used for this work. Mast cell imprints were made on the base foils covering the sample discs by pressing them against the mesentery. The loosely adherent mast cells, along with some others, readily came off the tissue surface and the imprints were dried at room temperature over calcium chloride *in vacuo*. After the X-ray photographs of the dried samples were taken, the cells were stained with toluidine blue for their metachromatic identification, Fig. 2 *c*, *d*. The

X-ray image of each cell to be measured and the corresponding step wedge in the original photomicrograph was projected (140 ×) on the glass screen of a photomicrographic camera, and densitometric measurements were made directly with a photomultiplier tube (Photovolt Corp., Model 520 M, Tube No. 21 C). The apparatus was set up in a manner similar to that employed by Lessler and Charipper [12]. To confine the light measured to the image of a cell, a hypodermic needle with its point sawed off was soldered at the syringe-end to an aperture cone which fitted over the window of the photomultiplier tube and the small open end of the needle was placed over the image. This arrangement is similar to that described by Ensgtröm *et al.* [8].

In order to calculate the mass per cell the geometry of the cell must be be known. Although the fresh cell in a physiological medium approximates a sphere, the drying required for the X-ray measurement causes shrinkage. The assumption was made for the calculations in Table I that this shrinkage is uniform in all dimensions so that the spherical form is retained.

RESULTS AND DISCUSSION

From Table I it appears that a significant but small increase in cell diameter was produced by the 48/80 treatments, and this was reflected in a decrease in cytoplasmic mass per unit area as measured by interference microscopy. Further comparison of the means of Groups I and II indicates no significant difference in mass per cell after the treatments. On the other hand, the cells from rat No. 9 had greater mass per unit area but the same mass per cell as those of Group I. The effect of 48/80 in causing disruption of peritoneal mast cells in the rat was not observed in these *in vitro* experiments.

The drying of the cells for X-ray absorption measurement, Table I, decreased the overall diameter to 39 per cent of its value in Hanks' solution. Incidentally, the data from rat No. 9 again illustrate the rather good agreement between interference and X-ray absorption measurements which had been pointed out previously [5].

The data for total mass per cell given in Table I were all calculated on the basis of a spherical cell. All of the evidence at hand indicates that the mast cell in Hanks' solution is spherical. If the shape of the cell were to deviate from a sphere, the values would become greater, e.g., calculated for a disc of the same diameter, the values would be 1.5 times those given in the table. The actual shape of the dried cells used for the X-ray measurements is not known, but the assumption that they are spherical gives data which is most in accord with those obtained by the interference method.

SUMMARY

Both interference microscopy and X-ray absorption measurements were made of the dry mass of mast cells from the peritoneal cavity of the rat. On cells isolated in a sucrose density gradient and transferred to Hanks' solution, interference measurements on each of 97 cells from 4 rats gave a corrected mean dry mass per unit area of 8.8 ± 0.2 (g $10^{-13}/\mu^2$), and, calculated for spherical cells, a corrected mean total dry mass per cell of 2.0 ± 0.1 (g 10^{-10}). In another group of 81 cells from 4 different rats, the cells were treated *in vitro* with 1 to 3 μg/ml of the histamine liberator, 48/80. Corresponding data for this group were found to be 7.3 ± 0.1 and 1.8 ± 0.1, respectively. The difference between the mean values of dry mass per unit area of the two groups is significant, but for total dry mass per cell it is not.

Both interference and X-ray absorption mesurements were made on peritoneal mast cells from the same rat. The corrected mean dry mass per unit area by interferometry of 15 cells was 9.8 ± 0.5 (g $10^{-13}/\mu^2$), which is also greater than the corresponding value of the cells treated with 48/80. However, the corrected mean total dry mass per cell was 2.0 ± 0.1 (g 10^{-10}) which is close to the corresponding mean value of the cells treated with 48/80. Thus it appears that 48/80 *in vitro* does not cause loss of dry mass from those mast cells which remain intact. The mean total dry mass per cell obtained from the X-ray measurements, 2.3 ± 0.1 (g 10^{-10}), was fairly close to the value obtained by interferometry, 2.0 ± 0.1 (g 10^{-10}).

Phase contrast and interference microscopy indicated that the mast cell in Hanks' solution is spherical with a central spherical nucleus. The density of the latter appears to be less than that of the surrounding cytoplasm.

The authors wish to express their appreciation for the technical assistance given by Gunhild Ottoson and Donna DenBoer.

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THE NUCLEIC ACID AND PROTEIN CONTENT OF ROOT TIP CELLS OF *VICIA FABA* AND *ALLIUM CEPA*

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Received September 16, 1957

The importance and nature of the role of the nucleic acids, particularly as related to protein metabolism, in cell development is a question of great current interest. As has been pointed out repeatedly [1, 6, 7, 9], the root tip offers an unusual opportunity to study the chemistry and physiology of cell development by enabling morphological and biochemical data to be combined on a cellular level. There are several means by which such studies can be made; the technique used determines the type and extent of the data collected and influences the interpretation of the results.

Comparative studies are useful in attempting to formulate generalized patterns and it is for this reason that the root tip of *Allium cepa* (onion) was analyzed morphologically by the same techniques as were used earlier with root tips of *Vicia faba* (broad bean). This report carries this comparative analysis to a biochemical level by reporting the results of determination of deoxyribosenucleic acid (DNA), ribosenucleic acid (RNA) and protein per cell for the first twenty 100 micron sections of the root tip of *A. cepa*. A short report on the determinations of DNA and RNA in the first 3 mm of *V. faba* was published earlier [10] but an error in the ordinate of the graph presented makes it necessary to present the data again.

MATERIALS AND METHODS

Sets of *Allium cepa* var. white globe were used as the source of all onion roots. The sets were placed in vials of tap water and roots appeared in two to three days. The roots used were between 10–15 mm in length and were selected carefully for uniformity. The first 5 mm were excised and frozen in isopentane cooled with liquid nitrogen, dried in a moving gas freeze-dry apparatus, and paraffin infiltrated and imbedded. The roots were carefully aligned in the paraffin so that two or three could be sectioned at one time. The roots were sectioned at 50 microns and two such sections

[1] This work was supported by a grant from the National Cancer Institute of the U.S. Public Health Service.

or 100 microns of tissue were placed in small tubes 2 × 20 mm. The paraffin was removed with toluene and the tissue rinsed with 100 per cent ethyl alcohol. The procedures used for the *V. faba* root tips have been described [10].

The nucleic acids were extracted with perchloric acid and measured by ultraviolet absorption. The technique used was the standard Ogur-Rosen procedure modified to a histochemical level [10]. Nine roots were necessary for each determination with six sets of determinations being made. Absorption curves were made on extracts from both the *V. faba* and the *A. cepa* roots at various levels.

Protein was precipitated with hot alcohol and nitrogen content was determined by Nesslerization after digestion. This procedure was identical to the method of Jensen [9] except that 0.04 ml digestion mixture was used instead of 0.4 ml. Three roots were necessary for a determination and six sets of determinations were made.

The methods used in determining cell number, in both *V. faba* and *A. cepa*, have been described in detail elsewhere [9, 12]. Briefly, the method consists of making a histological analysis of the area and then selecting sections which are morphologically distinct. These sections are analyzed for the volume occupied by each tissue and the average cell length, cross-sectional area, and volume of the cells of each tissue. Cell number per tissue and per section can then be calculated. The method, while laborious and time consuming, offers a detailed, quantitative picture of cell development.

RESULTS

The amount of DNA, RNA, and protein per section and per cell of root tip of *A. cepa* together with the amount of DNA and RNA per cell of *V. faba* are presented in Table I. The amounts per cell together with diagrams of the roots are presented in Fig. 1. The two graphs have the same scale on the ordinates but different scales on the abscissae. The diagrams of the roots are on the same scale as the abscissae of the graphs.

Morphology.—Complete morphological analyses of *V. faba* and *A. cepa* have been reported and discussed elsewhere [9, 12] and will not be repeated here. To interpret the nucleic acid and protein data, however, it is necessary to have a general picture of cell development and root organization.

The area of the root tip under consideration here, 3 mm in bean and 2 mm in onion, is highly organized and contains several phases of cell development. In both roots, immediately behind the root cap, there is a group of cells, more highly organized in *A. cepa* than in *V. faba*, which may be termed the apical initial group [5] or, more loosely, the general meristem. It is this group of cells which the work of Clowes [3, 4] and Jensen and Kavaljian [12] indicates is either not dividing or possesses a very low rate of division. This group of essentially non-dividing, non-developing cells constitutes a significant portion of certain sections of each root (section #3 in *V. faba* and #5 in *A. cepa*, Table I). Immediately behind this apical initial group, the cells

TABLE I. *Nucleic acid content of root tips.*

Amount per section expressed as 10^{-8} g; amount per cell as 10^{-12} g.

	Vicia faba					*Allium cepa*				
		DNA		RNA			DNA		RNA	
Sec.[a]	Dist.[b]	Sec.	Cell[c]	Sec.	Cell	Dist.[b]	Sec.	Cell[c]	Sec.	Cell
1	100	--[d]	—	—	—	50	—	—	—	—
2	300	0.8	30	2.8	49	150	—	—	—	—
3	500	1.7	18	4.4	40	250	—	—	—	—
4	700	4.2	35	10.4	102	350	—	—	—	—
5	900	6.7	52	14.0	136	450	1.9	40	4.5	94
6	1100	7.6	56	20.8	179	550	2.2	41	7.1	134
7	1300	8.6	61	26.4	216	650	2.8	51	9.0	164
8	1500	8.4	58	29.2	226	750	4.2	76	10.7	193
9	1700	9.4	63	31.6	231	850	4.7	85	13.6	246
10	1900	9.8	65	34.0	253	950	5.2	95	16.1	293
11	2100	9.4	61	35.6	248	1050	5.0	93	17.4	321
12	2300	10.2	66	35.6	251	1150	4.5	86	16.3	310
13	2500	9.0	60	34.0	248	1250	4.1	82	14.0	280
14	2700	8.0	55	34.8	236	1350	4.0	84	14.0	294
15	2900	9.5	64	33.6	247	1450	4.1	93	13.3	302
16						1550	3.8	94	12.9	318
17						1650	3.3	91	11.0	301
18						1750	3.1	97	8.0	250
19						1850	2.7	98	7.7	280
20						1950	1.9	84	7.3	317

[a] Sections were 200 microns thick in *V. faba* and 100 microns thick in *A. cepa*.
[b] Distance from tip to midpoint of section.
[c] See Jensen [9] and Jensen and Kavaljian [12] for number of cells per section.
[d] — indicates that the per section content was below the limits of the technique.

begin rapid radial enlargement. The cells of the various tissues have different rates of enlargement and different final diameters. It is these differences which contribute to the primary morphological differentiation of cell types. During the period of maximum radial enlargement, the mean cell length is constant and it is only as the final cross-sectional area of the cell is reached that elongation begins. The increase in cell length is at first small but becomes greater in the final sections. It is believed, however, that the period of maximum elongation is not contained in the root length studied here.

All the cells present in this length of root are capable of cell division with the exception of the apical initial group and the extreme root cap, with the

maximum number of cell divisions occurring in and around the region where elongation begins. Careful analysis of the onion root tip indicates that the maximum number of divisions in the various tissues occurs in different zones. In the cortex, for example, the largest number was found shortly

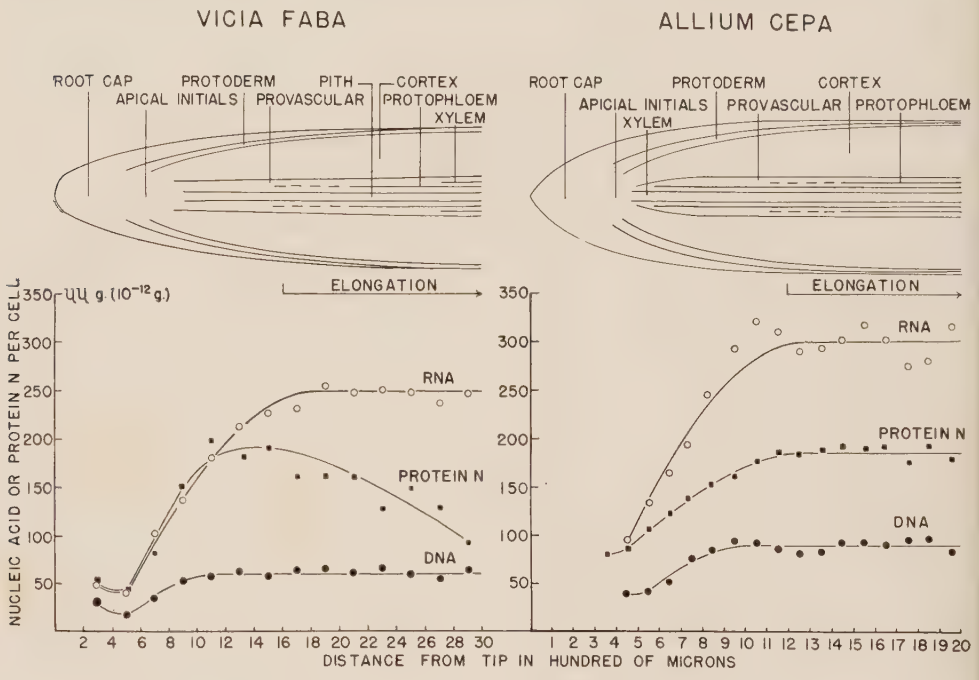

Fig. 1.—The amount of DNA, RNA, and protein N per cell of the root tip of *V. faba* and *A. cepa* as a function of distance of section analyzed from the tip of the root. The diagrams of the roots are on the same scale as the abscissae. The two graphs have the same scale on the ordinates but different scales on the abscissae.

before the beginning of elongation while in the vascular tissue the maximum occurred after the start of elongation [12]. The cells which were observed in division covered the complete range of morphological types and phases of development present, with the exception noted above. It is, consequently, impossible to define a distinct set of morphological or cytological characters that would denote a "meristematic cell". The concept of the "meristematic cell", i.e., a cell of distinct morphology, primarily engaged in division, and acting as the starting point or first phase of cell development, simply does not fit the data. Cell division appears to be rather a potentiality possessed by cells through the early stages of development and which is lost only as the

cell becomes fully elongated. This means, in effect, that studies with root tips can yield information on cell division only if cell division is treated as a process of cells of unlike morphology rather than a distinct phase of cell development. Attempting to use root tips in studies of the physiology and biochemistry of cell division can be meaningful only when the experiments can be analyzed in terms of differences in growth rates [6] or in which cell division cycles, as occur in *A. cepa*, can be utilized. These limitations obviously do not apply to experiments in which the mitoses are observed directly.

Deoxyribosenucleic acid (DNA).—The pattern of DNA content per cell as a function of distance from tip is very similar in *V. faba* and *A. cepa*. In both cases the lowest amount of DNA per cell occurs in the region of the apical initial group. The amount then increases until a constant value is reached several hundred microns later but preceding elongation. In *A. cepa* the higher value is almost precisely double the low amount, while in *V. faba* it is somewhat greater.

Various reports indicate that, in root tips, DNA doubling occurs some 6–8 hours [8, 12] before mitosis. As observed under the morphological results, the apical initial group is composed of cells which are either not dividing or which have a very low rate of division. The section in both roots which contains the cells with the lowest amount of DNA is also the section which contains the apical initials. This correlation suggests that the amount of DNA per cell in this section represents the DNA value of the 2n nucleus. The high DNA values would then be the result of the doubling of DNA in actively dividing cells and, possibly in polyploid nuclei.

The nucleic acid content of the *V. faba* root tip has also been determined by Holmes *et al.* [7] using sections of varying length, the shortest being 2 mm. They analyzed segments of the root which were 0–2, 2–4, 4–8, 12–20 mm from the base of the meristem and discarded the root cap. Cell number of each segment were determined by macerating the roots and counting the resulting cell suspension. By averaging the values obtained here from sections 3 through 13 (deleting sections 1 and 2 to adjust for the lack of root cap cells in the Holmes *et al.* figure) it is possible to compare, to a limited extent, the two sets of data. As Holmes *et al.* also measured phosphorus content it is necessary to use a conversion factor of 0.099 to convert DNAP to DNA. These calculations result in the following data:

	DNA	RNA	RNA/DNA
Holmes *et al.*	36×10^{-12} g/cell	144×10^{-12} g/cell	4.0
Present data	45×10^{-12} g/cell	188×10^{-12} g/cell	3.8

The variation in technique includes differences in the methods of both the morphological and biochemical analysis as well as differences in the varieties of *V. faba* used, yet the agreement is very good. This agreement indicates that the large segment analyzed by Holmes *et al.* contained the low value of the apical initial group which they failed to detect. It would appear then that the figures obtained by Holmes *et al.* more closely represents the 4n nucleus rather than, as they concluded, the 2n nucleus.

Ribose nucleic acid (RNA).—The pattern of RNA content per cell is identical in the two species and can be closely correlated with the morphological development of the cells. The apical initial group is low in RNA, while in the radially enlarging cell the increase in RNA is directly proportional to cell size. With elongation this increase in RNA ceases and RNA content remains constant during the beginning of elongation. From the work of Holmes *et al.* it would appear that there is a second rise during the period of maximum elongation followed by a decrease per cell as elongation terminates. Thus, while DNA content seems primarily a nuclear phenomenon and unrelated to cell size or shape, RNA is closely related to morphological development of the cell.

The amount of RNA per cell is different in the two species but the ratio of RNA:DNA is very similar; 4.1 in *V. faba* and 3.6 in *A. cepa*. The ratio is calculated from constant values present after 1500 microns in *V. faba* and 1200 microns in *A. cepa*. (The ratio for *V. faba* is slightly different from that given under the DNA section earlier because there the data from sections 3–13 were averaged.)

Protein N. -The pattern of protein per cell, unlike that of the nucleic acids, differs in *V. faba* and *A. cepa*. This difference, however, occurs only with cell elongation. In both species protein content is low in the apical initial group, increasing sharply during radial enlargement. With elongation the amount of protein per cell remains constant in *A. cepa* but decreases in *V. faba*. This pattern, except for the decrease in *V. faba*, is remarkably similar to that of RNA.

The protein content of the root tip of *V. faba* has also been measured by Robinson and Brown [14]. Using one millimeter sections, they expressed the data on a per cell basis after determining cell number by the Brown-Rickless maceration-haemocytometer technique [2]. This procedure has been criticized by Jensen [9] who compared data obtained by the two methods from two different species. It now appears that the difference in the species was greater than anticipated and the criticism unjustified. Allowing for differences in section size, growing conditions (which would most notably effect the

amount of root cap present), and varieties of *V. faba* used, the results obtained are in close agreement. Moreover, by combining the two sets of data, one collected from fifteen 200 micron sections and the other from fifteen one millimeter sections, it is clear that the protein content of the apical initial cell is very low and that there is a rapid increase during radial cell enlargement followed by a decrease as the cell begins elongation. As elongation continues [2], the protein content per cell again increases, this time to a much higher level until there is a second decrease roughly corresponding to the termination of elongation.

In the light of the *V. faba* results, the question arises as to the nature of the protein pattern in *A. cepa*. Does the value of protein per cell between 1200 and 2000 microns remain constant through the period of maximum elongation or is there a second increase as in *V. faba*? Unfortunately, there are no analyses of *A. cepa* similar to that of Robinson and Brown for *V. faba*. Erickson and Goddard [6], however, have measured protein content in the corn root tip over the first fifteen millimeters. While corn and onion differ considerably they are both monocotyledons with highly organized root tips of similar nature. The protein N per cell curve for the first two millimeters of corn and onion are very similar, both increasing rapidly and then leveling off at slightly over one millimeter from the tip and remaining constant for approximately 700 microns. At two millimeters from the tip the protein N per cell in corn again begins to increase with a flat peak being reached five millimeters from the tip, followed by a decrease in protein per cell before the termination of cell elongation.

Thus, the changing protein content in the developing cell shows a highly complicated pattern with two periods of increase, one accompanying radial enlargement, the other with elongation, separated by a period of either decrease or constancy at the beginning of elongation. This pattern is followed very closely by the RNA content of the cell. The period at the beginning of elongation appears to represent a distinct physiological stage in cell development.

DISCUSSION

The conception of cell development in the root tip has become increasingly complicated as data on the cellular level accumulates. By combining the data presented here with that of a number of other workers it is possible to draw a rather intriguing picture of the root tip.

The first cells encountered in the root tip are those of the root cap. These interesting cells, often overlooked, are remarkable for the short space in

which they develop and mature. They are low in protein and RNA, high in carbohydrates, with rather high oxygen uptake [9], and polyploid nuclei [15]; all the characteristics of fully mature cells. They should not be ignored in determinations involving sections, particularly where enzymes are being measured.

Immediately behind the root cap are the apical initials (general meristem [9]). These cells appear to be non-dividing [3, 4, 12] and are very low in protein, RNA, carbohydrates, oxygen uptake, and everything else measured. The DNA content of these cells is the lowest found in the root and assumed to be the amount found in the 2n nucleus. The role of these cells is completely unknown and they appear to be set apart from the normal pattern of cellular development.

The first stage of development appears to be one best described as the radial enlargement phase. Here cells are rapidly increasing in diameter and variations in the rate and degree of radial enlargement contribute markedly to the primary morphological differentiation of the tissues. Protein, RNA, DNA and carbohydrate content of the cells increase greatly during this stage although oxygen uptake remains low [9, 16]. As a result of the occurrence of frequent cell divisions and possible polyploidy the average DNA content per cell is almost double that of the apical initials.

Elongation, which is minimal during radial enlargement, begins as the cells reach their maximum diameter. From the data it is evident that cell elongation must be divided into several phases. The first, encompassing the beginning of elongation, is characterized by a low rate of elongation, frequent divisions, a leveling of RNA content, a leveling or decrease of protein content, an increase in carbohydrates but without a parallel increase in hexoses, an increase in oxygen uptake [1, 9, 16] and an increase in ascorbic acid with a shift of localization [11]. The second phase, that of maximal rate of elongation, can be characterized by a cessation of divisions, an increase in both RNA [7] and protein [1, 6], an increase in carbohydrate and high oxygen uptake [16]. The third and final phase is marked by a decreasing rate of elongation, and a decrease in RNA [7], protein [1, 6], and oxygen uptake. During the second and third phases the average DNA content per cell increases as a result of the presence of polyploid nuclei [7, 15].

A great deal more information must be obtained before a comprehensive picture of cell development can be drawn. Data is particularly needed from the earliest stages of development, as it is here that the blocking out of cell and tissue differentiation occurs. This information can be gained only from thin sections and a recognition of the cellular complexity of the area.

SUMMARY

The DNA, RNA, and protein content of the cells of the first two millimeters of *Allium cepa* (onion) and the first three millimeters of *Vicia faba* (broad bean) was determined. The data indicate that a group of cells, usually termed the apical initials or general meristem, are not dividing or dividing at a very slow rate. The value of DNA, RNA, and protein per cell for this group of cells is the lowest found in the root and it is believed that this DNA value represents that of the 2n nucleus. Basal to this area divisions occur frequently and the DNA value per cell doubles. The cells immediately basal to the apical initials enlarge radially and increase threefold in protein and RNA per cell. As radial enlargement ceases elongation begins and a leveling of RNA content per cell occurs. In *A. cepa* protein content per cell also levels while in *V. faba* there is a decrease in protein per cell. It is believed, however, that in both cases there is a second increase in protein associated with maximal elongation although that stage is not included in the work reported here.

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PROTEIN SYNTHESIS IN NEWT OOCYTES

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Received September 16, 1957

Where in the cell is new protein synthesized? This important question has been so far approached in several ways which fall into two groups. One comprises experiments on the living, normally synthesizing cell, the other on isolated nuclei or cytoplasmic particles *in vitro*.

Caspersson's school (see [1]) have investigated whole cells by sensitive spectrophotometric techniques and have derived from their work a theory concerning the role of the nucleolus in the synthetic activity of the cell. They credit this structure with supplying to the cytoplasm ribonucleic acids and proteins; in turn, the arrival of these substances in the cytoplasm is correlated with further protein synthesis there.

Other workers have used radioactive precursors of RNA and of protein to study the distribution of incorporated tracer in various parts of the cell by autoradiography. The work reported here is a contribution to this latter type of studies.

Experimental requirements.—In studies of this type, tracers such as P^{32} and C-labelled adenine are added to the medium for the purpose of labelling nucleic acid molecules whilst these are being synthesized. Amino acids containing radioactive carbon or sulphur atoms are similarly used to mark newly synthesized protein molecules. Radioactive iron has been used to label hemoglobin. The localization of molecules thus marked is studied in autoradiographs obtained from histological preparations.

Difficulties arise from two facts. Firstly, incorporation of a labelled amino acid into protein will occur to some extent, by exchange, even in the absence of net synthesis. Secondly, protein newly synthesized in one cell site might move to another, in which case it will be traced in the autoradiograph not at the site of synthesis but at some other site of deposition. For example, hemoglobin is probably formed in the nucleus [7] but accumulates in the cytoplasm of erythrocytes. In general, given time, the tracer will spread throughout the cell and a subsequent autoradiograph may provide no evidence as to the site of synthesis.

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For these reasons, the experimental procedure must be such as to show up the bulk of labelled protein while this still finds itself at the sites of synthesis. This initial autoradiograph might be obtainable by reducing the time intervening between the supply of tracer and the fixation of the tissue. This, again, conflicts with the requirement of a labelling period long enough to give a detectable autoradiograph.

Further, to detect any movements of labelled molecules away from the site of synthesis, autoradiographs from tissue samples fixed at various intervals should be compared. But such "time-lapse" autoradiographs will be very difficult to interpret if the cell is left throughout in the tracer. For, whilst the first synthesised labelled protein might be moving out of the site of synthesis, more tracer will be entering this continually, and will mask that movement. Therefore the tracer should only be available for a short labelling period and be withdrawn at the end of this.

These requirements have not been met in all investigations of this type; thus, chick embryos have been exposed by some authors to the tracer for 9 hours [10], amphibian embryos for 1 to 3 days [13], and frog oocytes for 1 or more days [3, 5]. No conclusions regarding the site of protein synthesis can be drawn with confidence from these experiments.

In contrast, the above requirements have been approximated in an investigation by Taylor, McMaster and Caluya [11]. These workers fed *Drosophila* larvae on food containing radioactive P^{32} for only one or two hours (labelling period) and then transferred them to unlabelled food (thus discontinuing the supply of tracer), and collected larvae for fixation at intervals. The cells of the salivary glands were studied by autoradiography and a large concentration of activity was detected in the first instance in the nucleolus-associated chromatin; subsequently the activity became relatively higher in the nucleolus and the cytoplasm. The results were interpreted as compatible with either of two hypotheses: that the nucleolar RNA is a precursor of cytoplasmic RNA, or that the nuclear RNA is a precursor of both.

Extension of similar work to several cell types would be interesting in that it might help to clarify the present picture of synthetic processes, and might also reveal differences between various cell types. The developing amphibian oocyte is a suitable cell in some respects, because of its size and the large-scale and uninterrupted [9] protein synthesis involved in vitellogenesis. Ficq [3] and Kemp [5] have worked with frog oocytes using as tracers carbon-labelled glycine, phenylalanine and adenine (Ficq) and glycine (Kemp). The present experiments were carried out on newt oocytes and were undertaken in an effort to satisfy more closely the requirements mentioned. To

TABLE I. *Particulars of experiments.*

Exp. no.	Tracer	Activity injected (μC)	Dilution	Fixation time from injection (hours)
		Triturus palmatus		
1	DL-methionine-S^{35}	5	—	4
2		5	—	8
3		5	—	24
		Triturus alpestris		
4	Glycine C^{14}	5	—	6
5		5	—	13
6		5	—	24
7	DL-methionine-S^{35}	10	—	1.5
8		10	At 1.5 hours	3
9		10	At 1.5 hours	6
10		10	At 1.5 hours	9
11		10	At 1.5 hours	19

this end, time-lapse autoradiography was combined with a brief labelling period and with a dilution of the labelled with unlabelled amino acid at the end of that period. A first experimental series aimed at discovering empirically suitable fixation intervals, and a second at providing a clear picture of the uptake pattern at all oocyte stages.

EXPERIMENTS AND RESULTS

First series

Female newts of the species *Triturus palmatus* and *alpestris* were injected at the end of the ovulation season in June 1956 with 50 units each of chorionic gonadotrophin; two days later they were injected with labelled glycine or methionine as specified in Table I. At the intervals listed, the ovaries were dissected and fixed in Zenker's fixative. Sections were cut at 6 μ and autoradiographs were obtained by the stripping film technique [2] after an exposure of 35 days.

It was found difficult to obtain good sections of the yolky oocytes (stage *D* described below) after fixation in Zenker's. Furthermore the yolky areas of these oocytes tend to darken soon after mounting. It was found helpful in this respect to treat the autoradiographs in a mixture of phenol and xylol (1:5) for hours before mounting and to replace Canada balsam with Michrome Cristalite.

Each ovary contained oocytes at all stages of development, exposed to the same treatment within the same animal. These stages can be specified as follows:

A, *Previtellogenetic stage.*—No nucleoli appear stained and there are no yolk platelets or pigment granules. The cytoplasm stains deeply red with pyronin.

B, *Late previtellogenetic stage.*—Pigment grains and yolk platelets are still absent, but numerous nucleoli make now their appearance and stain deeply red with pyronin.

C, *Vitellogenetic stage.*—Yolk platelets are being formed starting from the periphery of the cytoplasm. The pyronin is not taken up by the platelets but only by the cytoplasmic ground-substance between them.

D, *Mature oocyte stage.*—The whole extranuclear area is filled at this stage with platelets and with pyronin-positive ground-substance between them. There are abundant pigment grains in the outer cytoplasmic layers. The germinal vesicle lies eccentrically and is smaller, in relation to the whole cell, than at earlier stages. Many of the nucleoli are now found around the central area of the germinal vesicle.

It should be noted that the number of nucleoli in oocytes is large and that it increases still more in stage *D*. This in itself has raised doubts as to whether they are homologous with the nucleoli of somatic cells [4]. It is not in dispute, however, that they are rich in RNA.

Second series

The results of the first series were encouraging from the technical point of view, in that good autoradiographs could be obtained with brief labelling periods. In the second series of experiments (June, 1957) this period was further reduced, to 1.5 hours, but only methionine was used. Details of these experiments are included in Table I (exp. [7–11]). This time all the animals were further injected at the end of the labelling period of 1.5 hours with 0.5 cc of a solution (in Ringer's) of non-radioactive DL-methionine (6 mg per 1 cc, this being the concentration equivalent to that of the tracer solution). This treatment did not of course amount to a complete "withdrawal" from the circulation of any tracer not utilised within the labelling period, but it must have caused a drastic dilution of it.

Examination of autoradiographs

The comparisons made by visual examination were confirmed by detailed grain counts under an oil immersion lens. Generally, the agreement between counts was very good and each figure in Table III is the mean of 12 counts over areas of 52 μ^2. It should be noted that the activity accompanying the

TABLE II. *Relative activity concentrations in oocytes. First series of experiments.*

Cy = cytoplasm; N = nucleus; NA = nucleolar area.

Exp. no. and fixation time (hours)	Stage *A*			Stage *B*			Stage *C*			Stage *D*		
	Cy	N	NA	Cy	N	NA	Cy	N	NA	Cy	N	NA
1 (4)	++	+	—	++	+	+++	+	+	+++	+	+	++
2 (8)	+	++	—	++	++	+++				++	+	++
3 (24)	+	+	—	++	+	+++				++	+	+++
4 (6)	+	+	—	++	++	+++	+ or ++[a]	+	+++	+	++	+++
5 (13)	+	++	—	+	++	+++				+	+	++
6 (24)	+	++	—	+	++	+++				+	+	+++

[a] +, perinuclear cytoplasmic zone; + +, yolk platelet (outer cytoplasmic) zone.

nucleolus is localized not only within this structure itself but also around it. Therefore the counts were made over the whole nucleolar area. Agreement between counts over these nucleolar areas was less good in 4 cases which are marked by asterisks in Table III; the standard error in these is rather large. This lack of uniformity in the labelling of nucleoli at certain stages is being considered in the discussion.

The results of the first, exploratory, experimental series are given only in a qualitative form (Table II). Those of the second series are given numerically in Table III, and as percentages—counting the sum of the means for cytoplasm, nucleus and nucleolus as 100—in Table IV.

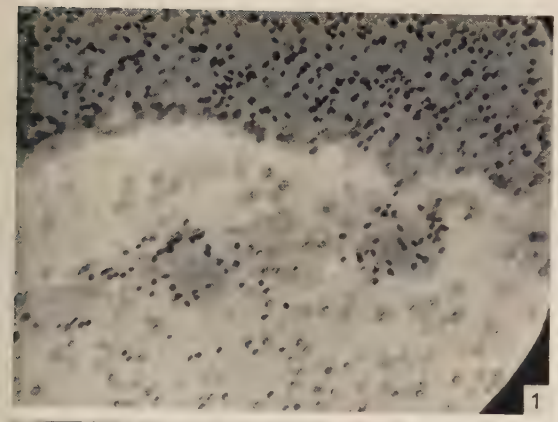
Fig. 1.—Autoradiograph of a stage *B* oocyte from exp. 7. The microphotograph includes only a small part of the oocyte, covering some cytoplasm (towards the top of the page, deeply stained with pyronin) and a corner of the germinal vesicle (practically unstained). Two deeply stained nucleoli are included in this latter part. The density of grains over the cytoplasm exceeds that over the nucleus. The nucleolar areas have no higher density than the cytoplasm, in fact grain counts show that they have less (see Table III).

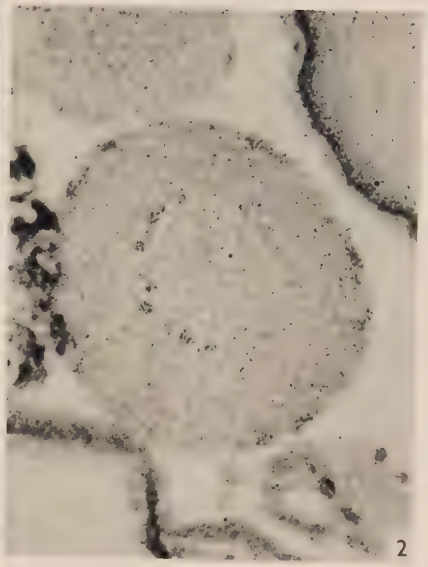
Fig. 2.—Autoradiograph of a stage *B* oocyte from exp. 3, i.e. after a long labelling period. In contrast to Fig. 1, the nucleoli have a higher activity than the cytoplasm. Again, the nucleus shows the smallest grain density.

Fig. 3.—High power photograph of a part of the autoradiograph illustrated in Fig. 2. The high activity in the nucleolar areas is shown. The nucleus (right side) has less activity than the cytoplasm (left). Note that the grains in the cytoplasm are localized around the yolk platelets but not within them.

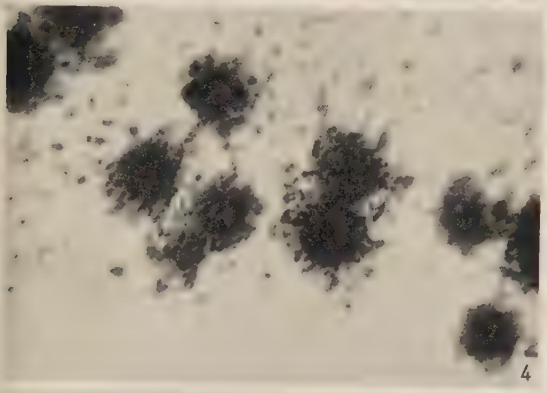
Fig. 4.—High power photograph of part of an autoradiograph over the germinal vesicle of a stage *D* oocyte from exp. 11. The nucleoli shown (deeply stained) belong to the group found in the central area of the germinal vesicle. In contrast to the germinal vesicle as a whole, the nucleolar areas show a very high density of grains.

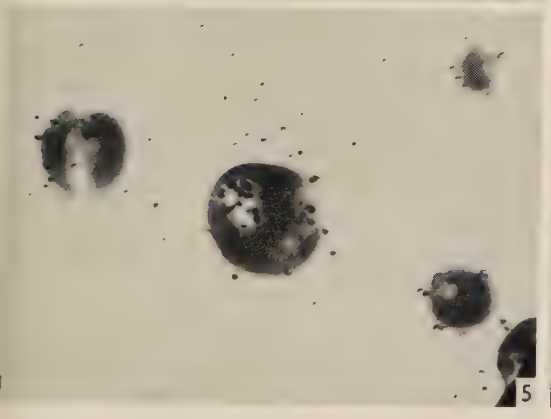
Fig. 5.—Part of an autoradiograph over the germinal vesicle, very weakly labelled, of a stage *D* oocyte. Four entire nucleoli are shown. Activity within the largest nucleolus is clearly concentrated in a large vacuole which does not stain with pyronin.


1


2

3


4


5

The type of autoradiographic pictures obtained is illustrated by Figs. 1 to 5. Fig. 2, taken at a low magnification, shows that the contrast in grain density between nucleoli, cytoplasm and nuclei is clear enough to allow a qualitative grading by visual inspection. The others, taken under an oil immersion lens, show that at the same time this density was such as to allow reliable counting, since the grains do not overlap to form thick clusters.

Localization was of course greatly helped by the large size of the oocytes and their constituent structures. This allowed, incidentally, the following observations regarding localization *within the nucleolus*. The nucleoli stain deeply red with pyronin, except for one or more vacuoles which they contain. It was noted that activity distribution in the nucleolus can assume several distinct patterns. For example, a nucleolus with a single large vacuole might have all its activity concentrated in the vacuole only (as shown in Fig. 5), suggesting perhaps that the vacuole is the site of accumulation of protein ready for delivery to the cytoplasm. This possibility of obtaining clear grain localization within a nucleolus is being used in a study now in progress.

DISCUSSION

The effect of experimental conditions.—A comparison should first be made between the results of the two series of methionine experiments. The uptake pattern in exp. 1 agrees more with that in exp. 8 than with 7. For example, in 1 and 8 the nucleolar area exceeds the cytoplasm at stage *B*, whilst in exp. 7 the reverse is true. By comparing fixation times in these three experiments it will be appreciated how the initial pattern can be masked by late fixation. Similarly, at stage *C* and at the end of the labelling period (exp. 7), the nucleolus by far exceeds the cytoplasm and nucleus, but later following the "dilution" (exp. 8), the level of the cytoplasm rises to equal it. This change, which could be reflecting an "emptying" of the nucleolus into the cytoplasm, would, but for the dilution injection, remain undetectable as it remained in exp. 2. A similar comparison can be made between exp. 11 and 3 (19 hours dilution, and 24 hours no dilution). In the former, the cytoplasm at stage *D* exceeds the nucleus whilst in the latter the reverse is true.

The above comparisons underline the usefulness of the dilution procedure and make it probable that a still clearer picture will be obtained by further approximations to a complete withdrawal of unused tracer at the end of a very brief labelling period.

Comparisons between different amino acids.—Glycine (exp. 4 to 6) gives in some respects a picture different from methionine. The nucleus takes up at

TABLE III. *Mean grain counts per 52 μ^2.*

Cy = cytoplasm; N = nucleus; NA = nucleolar area.

and time s)	Stage *A*			Stage *B*			Stage *C*			Stage *D*		
	Cy	N	NA	Cy	N	NA	Cy	N	NA	Cy	N	NA
5)	62±6	19±2	—	60±5	22±2	38±14	31±8	24±2	51±16*	32±7	18±3	54±10
)	43±5	25±1	—	61±8	17±3	86±11	60±7	18±2	58±16*			
)	36±7	18±1	—	60±3	19±3	49± 5	45±4	19±5	61± 7	41±9	21±2	51± 8
)	3±.05	1.5±.06	—	22±4	13±1	47±30	13±3	4±2	10± 5*	9 ⊦5	9±2	18± 2
)	7±2	2±.05	—	7±2	7±1	39±14	23±1	5±1	11± 4*	3±1	5±2	13± 2

least as much tracer as the cytoplasm; there is an exception, however, the yolk platelet zone of stage C exceeding the uptake of the perinuclear cytoplasmic zone. Both the rule and the exception can be accounted for by the fact that glycine is a precursor of nucleic acids as well as proteins. Normally, the nucleus shows more activity because of its high nucleic acid synthesis, but the yolk platelet zone of the cytoplasm exceeds it because it is a zone of intense protein synthesis.

The nucleolar area shows consistently the highest uptake. The fact that stage A has a marked uptake of glycine conflicts with Kemp's finding [5] that, 2 days after injection, the pre-yolk stages of frog oocytes showed no glycine uptake. This discrepancy could be explained by a lack of reaction to the gonadotrophin injection by the early frog oocytes.

The present experiments are not strictly comparable with those of Ficq [3] who studied the frog oocyte 24 hours after an injection of labelled phenylalanine. "Medium" and "large" oocytes showed an activity concentration in the nucleus twice as large as in the cytoplasm. This contrasts with our finding (with methionine) that the concentration in the cytoplasm equals or exceeds that in the nucleus. A comparative study of uptake of these two amino acids, which are both considered quite specific markers for protein, should be of interest.

Comparisons of the initial patterns of various oocyte stages.—The results of exp. 7, as set out in Tables III and IV, show throughout a lower relative activity for the nucleus than for the cytoplasm and the nucleolar areas; this applies to all four stages of oocyte development.

Comparing the last two cell components, one notes a difference between oocyte stages. At stage *B* the cytoplasm exceeds substantially the nucleolus;

TABLE IV. *Relative grain densities. (Mean grain counts as percentages of the sum of the means for the nucleus, the nucleolar areas and the cytoplasm.)*

Cy = Cytoplasm; N = nucleus; NA = nucleolar area.

Exp. no. and fixation time (hours)	Stage *A*			Stage *B*			Stage *C*			Stage *D*		
	Cy	N	NA	Cy	N	NA	Cy	N	NA	Cy	N	NA
7 (1.5)	77	23	—	50	18	32	29	23	48	31	17	52
8 (3)	64	36	—	37	10	53	44	13	43			
9 (6)	65	35	—	47	15	38	36	15	49	37	19	44
10 (9)	67	33	—	27	16	57	48	15	37	25	25	50
11 (19)	78	22	—	13	13	74	59	13	28	14	24	62

at stages *C* and *D* (vitellogenetic and mature), the nucleolar areas markedly exceed the cytoplasm. This might be taken to mean one of several things: firstly, that the nucleoli at vitellogenetic stages become more active than at stage *B*; secondly, that the nucleolar uptake does not change, but the cytoplasmic activity becomes reduced; thirdly, that the decrease in cytoplasmic activity is an artefact, due to the dispersal of the active cytoplasm between masses of inert yolk platelets synthesized already before the labelling experiment. Were the counts for cytoplasm to be made only over the basophilic reticulum between the platelets (and not over the cytoplasmic area as a whole), there is little doubt that the relative activity of the cytoplasm in Table IV for stages *C* and *D* would be much higher than for stage *B*. The first and second of these three possibilities would be consistent with the hypothesis that vitellogenesis is directly associated with a simultaneous high turnover of material in the nucleolar areas. The third possibility would be more compatible with the hypothesis of a local synthesis of yolk proteins in the cytoplasm.

Similar alternative interpretations apply to any cells which produce synthetic products in appreciable amounts and store them in the cytoplasm, and also to cells that become vacuolated. The salivary gland cells of *Drosophila* fall in this category, but this aspect is not discussed in the above-mentioned paper of Taylor, McMaster and Caluya [11].

It is also noteworthy that the uptake is high in the cytoplasm even at stage A, i.e. protein synthesis there does not need to wait for the appearance of nucleoli.

In conclusion, a comparison of the initial patterns of uptake by oocytes

of various stages shows that previtellogenetic protein synthesis in the cytoplasm is independent of the presence of nucleoli, and provides no evidence for linking vitellogenesis with a genuine rise of the rate of amino acid uptake in the nucleus or the nucleolus.

Changes in the grain density pattern following the labelling period.—The initial uptake patterns derived from exp. 7 will now be followed in time, i.e. 3, 6, 9, and 19 hours after the end of the labelling period and the administration of the dilution injection.

The pattern remains unchanged in stage *A* oocytes, the cytoplasm always exceeding the nucleus.

In stage *B*, the relative activity of the nucleolar areas rises substantially after the labelling period and becomes doubled after 19 hours. As this happens after the dilution injection, the rise of activity cannot be due to new uptake; it cannot be due to a condensation of the nucleolar material either, as the size of the nucleoli is not being reduced and as, anyway, most of the activity is not inside but around the nucleoli. It would seem therefore fairly probable that the relative activity of nucleolar areas rises by some intracellular transfer of tracer-carrying molecules. It should be noted that in the same period, whilst the relative activity of the nucleus does not fall markedly, that of the cytoplasm falls to $\frac{1}{3}$ or less of its initial value. It would seem therefore probable that there is a net movement from the cytoplasm into the nucleus.

Stage *C* is the period of intense vitellogenesis and growth. Although growth should reduce the relative grain density in the cytoplasm, this does actually rise markedly, by a factor of 2 in 19 hours. This could be explained by a movement of labelled molecules from the nucleus to the cytoplasm. Such a movement could occur via the nucleoli; in fact, the relative activity accompanying these falls. The high standard error of its value (marked by asterisks in Table III) does not affect this conclusion, and should in fact be expected if the nucleoli were fixed whilst at various degrees of "emptying". Most nucleoli at this stage are actually found wedged between nucleus and cytoplasm, and whenever at fixation the nucleus shrinks, many of them are left trapped in the innermost cytoplasmic zone. Wittek [14] has described and illustrated the physical connexion established between oocyte nucleoli and the cytoplasm in some detail. Recently, Lettré [8] described actually observing nucleolar discharge in living cells of other tissues.

The figures for the post-vitellogenetic stage *D* show no clear trend of a change in the grain density pattern, the nucleolar activity remaining relatively high throughout the period tested.

The hypothesis of two-way transfer.—These results can be read as indicating

two waves of intracellular transfer: one from the cytoplasm to the nucleus before vitellogenesis, and one from the nucleus, probably via the nucleoli, to the cytoplasm during vitellogenesis. These movements may be distinct in time, or may be simultaneous, the autoradiographic picture reflecting in that case only the *net* transfer. Two such migrations in opposite direction are not *a priori* less plausible than the often advocated one-way protein migration from the nucleus to the cytoplasm. It might be worth noting that a two-way migration would fit well the theory proposed by Chantrenne [6] of a two-stage proteosynthetic process. Before vitellogenesis, the cytoplasm can be thought of as synthesizing locally some "primary protein" of a rather general type; this subsequently passes to the nucleus where it acquires the imprint of specificity, presumably in the nucleolar areas, where chromosomal products are believed to occur. The now specific protein would be returned via the nucleoli to the cytoplasm during vitellogenesis. It might be reasonable to connect the large number of nucleoli in the oocyte with the large-scale and quick "processing" of proteins which is required to keep up with vitellogenesis; in contrast, in somatic cells, the deposition of cytoplasmic proteins can be dealt with by a single nucleolus. This scheme implies that the nucleoli are sites of accumulation of proteins on their way to the cytoplasm, rather than sites of actual synthesis of protein [12]. Nucleolar RNA might be involved not with the first stage of protein production, that of synthesis, but with the second stage, that of imparting specificity, in the way suggested by Koritz and Chantrenne [6] from their study of amino acid incorporation into hemogolobin.

The above hypothesis of two-way transfer is pieced together from data obtained by autoradiography. However, the inherent limitations of this technique should be kept in mind.

Where autoradiographs indicate a transfer, for example from the cytoplasm to the nucleus, the molecules actually migrating could be either cytoplasmic protein as such or some products of its partial breakdown which could be reassembled into new protein at the nucleus. Autoradiography supplies no evidence as to which is the case.

Again, grain density in the autoradiograph reflects the quantity of labelled molecules per volume of sectioned material. Grain density, however, can also change without a net loss or increase of the amount present in the structure examined as a whole, by changes in the density of the material.

In conclusion, the present results lead to a hypothesis of macromolecular cross-migration between nucleus and cytoplasm, but this hypothesis will have to be tested further by techniques other than autoradiography.

SUMMARY

1. Autoradiographs of newt oocytes of all stages were obtained after short (1.5 hours and 3 hours) periods of labelling *in vivo* with DL-methionine-S^{35} or glycine-C^{44}. Further autoradiographs were obtained at subsequent intervals, in some cases after a "dilution" injection of unlabelled methionine.

2. The evidence obtained suggests that labelled molecules migrate at the previtellogenetic stage from the cytoplasm to the nucleus, accumulating especially at the nucleolar areas. During vitellogenesis a transfer from the nucleolar areas to the cytoplasm appears to occur. A hypothesis of macromolecular cross-migration between nucleus and cytoplasm is formulated, but it is stressed that it should be tested by other methods also.

3. The results are discussed in connection with those of other workers who have studied the frog oocyte, and are linked with the theory that protein production might comprise two distinct stages (synthesis and specificity); it is suggested that the nucleolar area might be concerned with the second stage only.

4. The possibility of autoradiographic localization *within* the oocyte nucleolus is noted and illustrated.

The author is thankful to Professor C. H. Waddington for his interest in this work and for valuable discussion of this paper, and to the Melville Trust for a Research Fellowship.

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BEHAVIOR OF ENZYMES INVOLVED IN GLUCOSE-6-PHOSPHATE UTILIZATION DURING 6-DAY FASTING

STUDY OF HEPATIC GLUCOSE-6-PHOSPHATASE, PHOSPHOHEXOSEISOMERASE AND PHOSPHOGLUCOMUTASE

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Received September 26, 1957

In the mammalian organism glucose circulates in the blood and cannot be metabolically utilized in this form. The only reaction glucose is known to undergo in the organism is its phosphorylation resulting in the formation of G-6-P.[2] In the liver cells G-6-P may be channelled into 4 different metabolic pathways by the activity of 4 enzymes. (1) *G-6-Pase* G-6-P may be hydrolyzed by G-6-Pase and thus glucose is made available for the other cells of the organism. (2) *Phosphoglucomutase*—G-6-P may be routed into storage as glycogen and phosphoglucomutase is the enzyme which channels it into this pathway. (3) *Phosphohexoseisomerase*—G-6-P may be converted by this enzyme into F-6-P and this way it may enter into the glycolytic pathway yielding energy for the specialized functions of the cells. (4) *G-6-P dehydrogenase*—G-6-P may be directly oxidized by this enzyme and metabolized via the hexose monophosphate shunt. Thus it is clear that G-6-P occupies an important position in carbohydrate metabolism.

The metabolism of G-6-P has been under investigation in various centres. In our laboratories in the past few years the metabolic fate of G-6-P has been investigated by studying the physiological and pathological behavior of the 4 enzymes which are directly involved in G-6-P utilization. Weber and Cantero investigated the activities of these enzymes in normal, regenerating [17, 13, 23], embryonic, and newborn rat liver [17, 23, 24], Novikoff hepatoma [18, 19, 21, 24] and in various other tumors [18]. The effect of carcinogenesis was studied and the enzymes were assayed in liver of tumor-bearing animals

[1] Senior Fellow of the Cancer Research Society.

[2] The following abbreviations are used. G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; G-6-Pase: glucose-6-phosphatase.

[18]. Hormonal effects were examined in hypophysectomized and adrenalectomized animals [20, 28]. The effect of cortisone administration was studied (14, 15]. Recently the *in vitro* and *in vivo* effects of x-irradiation on these 4 enzymes were reported [25].

During the investigation of the behavior of enzymes which are involved in G-6-P utilization, the effect of short-term fasting has repeatedly been studied. It was noted that the basis of expression is of great importance in interpreting enzymatic results in fasting experiments when there are marked changes in the size [15], cellularity [1], and nitrogen content of the liver [22]. The studies on the effect of 1-day fasting demonstrated that the marked increase in hepatic G-6-Pase activity as expressed per unit wet weight [2, 16] was caused, partly at least, by the decrease in liver mass and consequent increase in liver cellularity [15]. The activities of phosphoglucomutase, phosphohexoseisomerase and G-6-P dehydrogenase showed only apparent alterations when expressed per wet weight or per nitrogen basis. However, when the enzymatic activities were expressed per average cell, phosphoglucomutase and phosphohexoseisomerase activities showed a statistically significant decrease after 24-hour fasting [27]. These data demonstrated that the 4 enzymes involved in G-6-P utilization behaved differently after 1-day fasting and their activity did not mechanically change with the decreasing nitrogen content of the liver [22, 27].

Since it seemed that fasting may reveal differences in the physiological behavior and biochemical characteristics of the 4 enzymes which are involved in the utilization of the same substrate, the activities of these enzymes were investigated during a 6-day fasting period. Fasting in this context was used as a tool in studying the behavior of enzymes. However, it was also necessary to obtain this information because under various experimental circumstances it was necessary to fast animals before they were sacrificed. On the other hand, under certain conditions the animals did not eat, although food and water were offered *ad libitum*. This occurred in experimental situations which produce anorexia (partial hepatectomy [17], fast-growing tumors [24], irradiation). When biochemical determinations were carried out on tissues from these experimental animals it was necessary to separate the direct effect of the experimental conditions from that of the fasting [24, 25, 26].

The present paper describes the behavior of hepatic G-6-Pase, phosphoglucomutase and phosphohexoseisomerase during a 6-day fasting period. The effect of fasting on body weight, liver weight, liver/body weight ratio, liver cellularity, and nitrogen content is presented and the enzymatic alterations are interpreted in the light of these data.

EXPERIMENTAL METHODS

Preparation of animals.—Male Wistar rats of 200–250 g of weight were used. The animals were kept in separate cages and maintained on Purina Fox Chow and water *ad libitum*. Fasted animals received only water. The animals were sacrificed 1, 3 and 6 days after fasting began. Fed animals of the same weight group were used to obtain normal values. There were 6 animals in each group and the whole series of experiments was repeated with similar results. No animal died during the 6-day fasting period.

Preparation of liver homogenate and supernatant fluid.—The animals were stunned, decapitated, and bled. The livers were quickly excised, placed in beakers standing on cracked ice, chilled for 5 minutes, then blotted on filter paper and weighed. Livers were minced with scissors and 10 per cent homogenates were prepared in isotonic KCl. Homogenization was carried out with a teflon plastic pestle turning at about 600 r.p.m. for exactly 3 minutes. The supernatant fluid was obtained by centrifuging the liver homogenates at 100,000 *g* for 30 minutes at 0°C. A refrigerated Spinco Model L centrifuge was used.

Cellularity determination.—The cellularity of the liver was determined by counting the nuclei in the liver homogenate according to the method of Price and Laird [11] as modified in our laboratories [1, 28].

Biochemical determination.—Nitrogen content was determined by the micro-Kjeldahl procedure. G-6-Pase activity was assayed according to Cori and Cori [4] with slight modification [18]. The release of inorganic phosphate was assayed by the method of Fiske and Subbarow. The activity was measured in g of inorganic phosphate released in 15 minutes at 30°C. The phosphohexoseisomerase activity was measured in the homogenate according to Bruns and Hinsberg [3] as modified by Glock and McLean [6]. One unit is the quantity of enzyme which produces one μM of F-6-P per minute per g wet weight of tissue at 37°C. The phosphoglucomutase activity was measured according to Najjar [10] in the supernatant fluid by determining the decrease in acid-labile phosphate after a 3-minute hydrolysis in 5 N sulfuric acid at 100°C. One unit is the quantity of enzyme which catalyses the disappearance of 1 mg acid-labile phosphorus per 10 minutes per g wet weight of tissue at 37°C. The optical density determinations were done by the Beckman Quartz Spectrophotometer, Model DU, with glass cells No. 2097. Proportionality to time and amount of enzyme at optimal pH and substrate concentration was established for all 3 enzymes before the experiments reported in this paper were started.

Expression of cellularity and biochemical data.—The results of nuclear counts were expressed as cellularity indicating the number of liver cells in millions per g wet weight [12]. The term "total cellularity" means the number of cells in millions contained in the whole liver [27]. Nitrogen content was expressed as mg nitrogen per wet weight, per average cell, and per 100 g body weight. Enzymatic activities were expressed per unit tissue (per wet weight, per average cell) and per 100 g body weight (total available activity [27]).

Interpretation of enzymatic results.—It was emphasized previously in the 1-day fasting studies that in the interpretation of enzymatic alterations in starvation experiments, the basis on which the enzymatic activities are expressed is of special importance [22, 27]. The significance of the choice of basis for expressing enzymatic activities is further documented in the present paper. Enzymatic activity was expressed first of all, per unit wet weight. To counteract the changes in the size of the liver due to alterations in water, glycogen, protein, and fat content, the enzymatic activities were also expressed per average cell. To express the liver enzymatic activities in relation to the functional need of the animal, enzymatic activities were also expressed as total liver enzymatic activities per 100 g body weight (total available activity). In comparing the data of normal and fasting groups the results were expressed on a per cent basis when values of the control fed animals were taken arbitrarily as 100 per cent.

RESULTS

Changes in body weight, liver weight, liver/body weight ratio, and liver cellularity during 6-day fasting.—The body weight decreased to 93 per cent at 1 day, to 84 per cent at 3 days, and to 77 per cent at the end of the 6-day fasting. The liver weight decreased to 73 per cent at 1 day, to 56 per cent at 3 days, and reached 49 per cent at the end of the 6-day fasting period (Fig. 1). The liver/body weight ratio decreased to 78 per cent at 1 day, to 67 per cent at 3 days and was maintained at this level to the end of the 6-day fasting period (Fig. 1). On the other hand, the liver cellularity (number of cells per unit weight) showed a progressive increase during 6 days of fasting, presenting nearly a mirror picture of the changes in the liver weight (Fig. 1). The liver cellularity increased to 134 per cent at 1 day of fasting, further increased to 157 per cent at 3 days, and reached 174 per cent at the end of 6 days of fasting. The total cellularity, however, did not change during the 6-day fasting period (Table I).

Changes in liver homogenate and supernatant nitrogen content during 6-day fasting.—When the liver nitrogen content was expressed in the conventional way as mg nitrogen per unit wet weight, the following alterations were noted (Fig. 2). The nitrogen content of the liver homogenate increased to 110 per cent at 1 day, continued to increase to 120 per cent at 3 days, and fell back to 114 per cent after 6 days of fasting. On the other hand, the nitrogen content of the liver supernatant fluid apparently did not change during 6 days of fasting. However, when the nitrogen content was expressed per average cell it was shown that the liver nitrogen content steadily decreased during 6 days

TABLE I. *Total liver cellularity during 6-day fasting.*

Animals	Total cellularity[a] in millions
Normal fed	2198±245
1-day fasted	2023±135
3-day fasted	1782±137
6-day fasted	1794±158

[a] Mean of 6 or more animals and standard deviations are given.

of fasting (Fig. 3). The homogenate nitrogen content decreased to 82 per cent at 1 day, to 76 per cent at 3 days and to 65 per cent after 6 days of fasting. The supernatant nitrogen content decreased to 78 per cent at 1 day, to 64 per cent at 3 days and to 56 per cent after 6 days of fasting.

The nitrogen content per 100 g body weight also decreased progressively both in the homogenate and supernatant fluid (Fig. 4). The total available homogenate nitrogen content fell to 61 per cent at 1 day, to 50 per cent at

Fig. 1.—The effect of 6-day fasting on body weight, liver/body weight ratio, liver weight and cellularity.

Fig. 2.—The effect of 6-day fasting on liver homogenate and supernatant nitrogen content. The nitrogen content is calculated as mg per cent per g wet weight. The results are expressed in per cent, and the values of the normal fed animals are taken as 100 per cent. Homogenate nitrogen: • —— •; supernatant nitrogen: • – – – •.

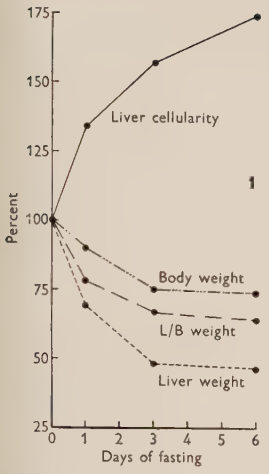
Fig. 3.—The effect of 6-day fasting on liver homogenate and supernatant nitrogen content. The nitrogen content is calculated per average cell. The results are expressed in per cent, and the values of the normal fed animals are taken as 100 per cent. Homogenate nitrogen: • —— •; supernatant nitrogen: • – – – •.

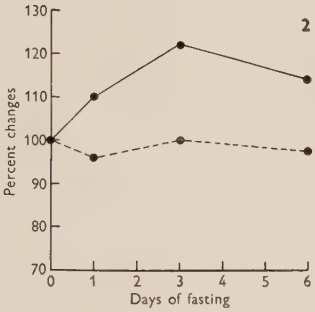
Fig. 4. The effect of 6-day fasting on liver homogenate and supernatant nitrogen content. The nitrogen content is calculated per 100 g body weight. The results are expressed in per cent, and the values of normal fed animals are taken as 100 per cent. Homogenate nitrogen: • —— •; supernatant nitrogen: • – – – •.

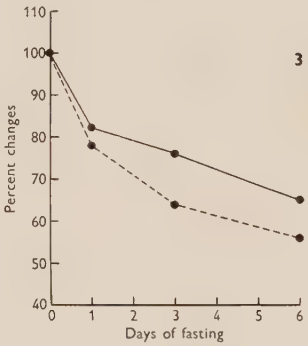
Fig. 5.—The effect of 6-day fasting on liver enzymes which take part in glucose-6-phosphate utilization. Enzymatic activities are calculated per g wet weight. The results are expressed in per cent, and the values of normal fed animals are taken as 100 per cent. *G-6-PASE*, glucose-6-phosphatase; *PHI*, phosphohexoseisomerase; *PGM*, phosphoglucomutase.

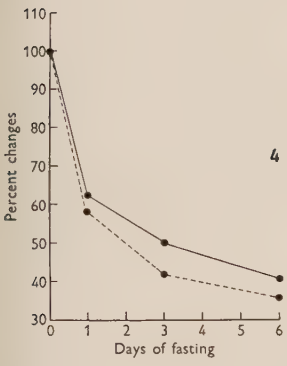
Fig. 6.—The effect of fasting on liver enzymes which take part in glucose-6-phosphate utilization. Enzymatic activities are calculated per average cell. The results are expressed in per cent, and the values of normal fed animals are taken as 100 per cent. *G-6-PASE*, glucose-6-phosphatase; *PHI*, phosphohexoseisomerase; *PGM*, phosphoglucomutase.

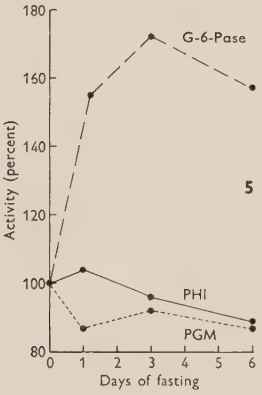
Fig. 7.—The effect of fasting on liver enzymes which take part in glucose-6-phosphate utilization. Enzymatic activities are calculated per 100 g body weight. The results are expressed in per cent, and values of the normal fed animals are taken as 100 per cent. *G-6-PASE*, glucose-6-phosphatase; *PHI*, phosphohexoseisomerase; *PGM*, phosphoglucomutase.

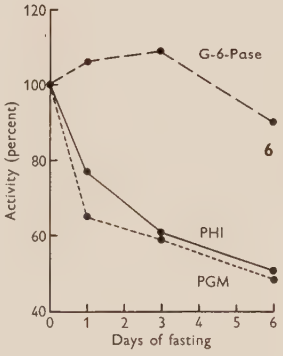

Liver cellularity
Body weight
L/B weight
Liver weight
Percent
Days of fasting
1

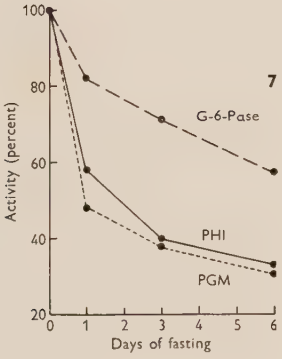

Percent changes
Days of fasting
2


Percent changes
Days of fasting
3


Percent changes
Days of fasting
4


G-6-Pase
PHI
PGM
Activity (percent)
Days of fasting
5


G-6-Pase
PHI
PGM
Activity (percent)
Days of fasting
6


G-6-Pase
PHI
PGM
Activity (percent)
Days of fasting
7

3 days and to 41 per cent after 6 days of fasting. The total available supernatant nitrogen decreased only to 58 per cent at 1 day, but fell sharply to 42 per cent at 3 days, and further decreased to 36 per cent after 6 days of fasting.

Enzymatic changes during 6-day fasting.—When the enzymatic results were expressed on various bases it was found that the marked shrinkage of the liver and the resulting crowding of the cells masked the alteration in enzymatic activities. The importance of finding the adequate basis for the expression of enzymatic activities when there are marked changes in the liver weight is shown in Figs. 5–7.

Changes in hepatic G-6-Pase activity during 6-day fasting.—When enzymatic activity was expressed on wet weight basis it was found that G-6-Pase activity increased to 155 per cent at 1 day, to 172 per cent at 3 days, and returned to 157 per cent at the end of 6 days of fasting (Fig. 5). The enzymatic activity was, however, in the normal range during 6 days of fasting in the average liver cell (Fig. 6). When enzymatic activity was expressed per 100 g body weight basis, it was found that the total available liver G-6-Pase activity gradually decreased during 6-day fasting: at 1 day it was 82 per cent, at 3 days, 71 per cent and at 6 days it went down to 57 per cent (Fig. 7).

Changes in hepatic phosphoglucomutase activity during 6-day fasting.—The liver phosphoglucomutase activity showed no significant changes from normal values when the enzymatic activity was expressed on wet weight basis (Fig. 5). However, when the enzymatic activity was expressed per average cell it was found that the phosphoglucomutase activity sharply decreased to 65 per cent at 1 day, then slowly levelled off reaching 51 per cent of normal value at the end of 6 days of fasting (Fig. 6). When phosphoglucomutase activity was expressed per 100 g body weight it was found that it decreased to 48 per cent at 1 day, to 38 per cent at 3 days and to 33 per cent at the end of 6 days of fasting (Fig. 7).

Changes in hepatic phosphohexoseisomerase activity during 6-day fasting.—When phosphohexoseisomerase was expressed on wet weight basis, the enzymatic activity was found in the normal range during the 6-day fasting period (Fig. 5). However, when enzymatic activities were expressed per average cell basis it was found that phosphohexoseisomerase decreased to 77 per cent at 1 day, went down to 61 per cent at 3 days and reached 49 per cent of normal values at the end of the 6-day fasting period (Fig. 6). When phosphohexoseisomerase activity was expressed per 100 g body weight it was found that the enzymatic activity decreased to 58 per cent at 1 day, to 40 per cent at 3 days, and it was 31 per cent at the end of the 6-day fasting period (Fig. 7).

TABLE II. *Amount of glucose-6-phosphate metabolized per hour at 37°C under optimal pH and substrate conditions.*

Enzymes	$\mu M \times 10^6$ per average cell	μM per 100 g body weight
Phosphohexoseisomerase	59	50,076
Phosphoglucomutase	28	25,802
G-6-Pase	2	1,782

Amount of G-6-P metabolized in vitro under optimal pH and substrate conditions.—The different behavior of the enzymes concerned with G-6-P utilization led us to calculate the amount of G-6-P which can be formed (phosphoglucomutase, phosphohexoseisomerase) or hydrolyzed (G-6-Pase) by the liver homogenate or supernatant fluid. Table II presents a summary of the amounts of G-6-P metabolized *in vitro* under optimal pH and substrate conditions. The results were expressed in μmoles per hour, per average cell $\times 10^{-6}$ and per 100 g body weight. The data show that phosphohexoseisomerase produced 59 μM, and phosphoglucomutase, 28 μM/hour/cell $\times 10^{-6}$. On the basis of 100 g body weight, this means 50,076 μM and 25,802 μM/hour respectively. On the other hand, G-6-Pase hydrolyzed only 2 μM/hour/cell $\times 10^{-6}$ or 1782 μM/hour/100 g body weight.

DISCUSSION

The experimental results presented in this paper demonstrate that the 3 examined hepatic enzymes which are involved in G-6-P utilization behave differently during 6-day fasting. These studies also illustrate the necessity of careful consideration and selection of an adequate basis for the expression of biochemical data when fasting conditions are used.

The effect of 6-day fasting on body weight, liver/body weight ratio, liver weight, cellularity, and nitrogen content (Figs. 2–4).—During 6-day fasting the animals lost 23 per cent of their body weight. It is important to note that no rat died during this period and that the animals were in good condition when sacrificed. Therefore, in these experiments, the effects of fasting were probably not influenced by possible non-specific changes which arise in moribund animals.

The liver weight progressively decreased to half the normal value at the

end of 6 days of fasting. As shown by actual cell counts, this marked shrinkage of the liver mass resulted in a concentration of the cells per unit liver. However, there was no loss of cells since the total number of liver cells (total cellularity) did not change. The concentration of the liver cell was higher than would have been expected on the basis of the decrease in liver weight. This may be interpreted as a decrease in both extracellular and intracellular constituents. The decrease in liver weight is probably chiefly due to loss of water, glycogen, fat and protein [7].

Unless the marked concentration of liver cells is taken into account, the results of biochemical determinations can be easily misinterpreted. This is clearly demonstrated in the analysis of nitrogen and enzymatic results. The impression gained when the nitrogen content of the liver homogenate was expressed per g wet weight was that this liver constituent markedly increased during 6-day fasting. This erroneous impression was due to the fact that the crowding of the cells masked the loss of nitrogen. However, when the nitrogen content was calculated per average liver cell, a marked progressive decrease was revealed.

When expressed on wet weight basis, the nitrogen content of the supernatant fluid seemingly did not change during 6-day fasting. However, a marked progressive decrease was observed when the values were expressed on per cell basis. The expression of nitrogen data on per average cell basis also shows that the decrease in the supernatant nitrogen is more marked than that of the total homogenate. This may be interpreted as an indication that the main decrease in the nitrogen content of the liver cells took place in the cytoplasm. Such a conclusion agrees with that of Harrison [7].

It is probable that most of the protein of the liver is in the form of enzymes. It is interesting from this point of view that the total liver nitrogen available for the body may decrease to 38–45 per cent of normal values while the animals remain in apparently good condition.

The effect of 6-day fasting on liver enzymes involved in G-6-P utilization (Figs. 5–7).—The shrinkage of the liver mass also masked the alterations in the enzymatic activities examined during 6-day fasting. When expressed on wet weight basis, G-6-Pase activity showed a marked increase, whereas phosphohexoseisomerase and phosphoglucomutase were essentially unchanged. However, when enzymatic activities were expressed per average cell, it was found that G-6-Pase activity was maintained in the normal range at 1, 3 and 6 days of fasting. On the other hand, both phosphohexoseisomerase and phosphoglucomutase progressively decreased to approximately 50 per cent of normal values at the end of 6 days of fasting. A difference in the be-

havior of these enzymes was also shown when total available activities were calculated. During the prolonged fasting phosphohexoseisomerase and phosphoglucomutase diminished to one-third of control values; G-6-Pase activity decreased to only 57 per cent of control values.

The different intracellular localization of the 3 enzymes which take part in the utilization of G-6-P may have a role in the diverse behavior of these enzymes during the 6-day fasting period. Phosphohexoseisomerase and phosphoglucomutase are supernatant enzymes [9], whereas G-6-Pase is associated with the microsomal fraction [8, 15]. G-6-P dehydrogenase, which is also a supernatant enzyme, was shown by Glock and McLean [5] to decrease very markedly during prolonged fasting.

Another important point in understanding the different behavior of the 3 enzymes which utilize the same substrate, G-6-P, is a consideration of the physiological function and the speed of the enzymatic reaction. In the fasting animals, the liver, which releases glucose by the G-6-Pase reaction, is the only source of blood sugar. Indeed, a close correlation between blood sugar level and liver G-6-Pase activity was demonstrated previously in fasting animals [20, 28]. Therefore, it is understandable that even in prolonged fasting G-6-Pase activity is maintained in near normal range. On the other hand, the pronounced decrease of phosphoglucomutase activity may be connected with the marked depletion of liver glycogen during fasting.

It is especially interesting to consider the decreased phosphohexoseisomerase activity from a physiological point of view. Under fasting conditions, carbohydrate is formed from fat and protein through the process of gluconeogenesis. These compounds must be broken down and go up through the reversed glycolysis eventually forming G-6-P. Since phosphohexoseisomerase is the enzyme immediately involved in the formation of G-6-P from F-6-P, this reaction might be expected also to be maintained on a normal level. However, the data showed that phosphohexoseisomerase was markedly decreased, finally losing two-thirds of normal activity. An explanation of the dispensability of part of the phosphohexoseisomerase activity may be found in Table I. It is clear from this Table that G-6-Pase activity is the slowest reaction of these 3 enzymes involved in G-6-P utilization. It is also obvious that, even when decreased to one-third of normal value, phosphohexoseisomerase would still be able to produce ten times more (19.4 moles) G-6-P than hepatic G-6-Pase would be able to hydrolyse (2 moles).

Statistical significance and biological significance.—The presented enzymatic data draw attention to the importance of the consideration of biological significance in addition to the indications given by statistical interpretation

[15]. The phosphohexoseisomerase results suggest that a marked excess of this enzyme is present in the liver cell; even a loss of two-thirds of the activity is compatible with adequate maintenance of physiological function. Therefore, although phosphohexoseisomerase showed a statistically significant decrease during 6-day fasting, still there was no physiologically significant decrease present because the enzyme was able to produce ten times as much G-6-P as could possibly have been hydrolyzed by G-6-Pase. This excess of G-6-P production may be correlated with the fact that the liver glycogen stores are not completely devoid of glycogen even after 6 days of fasting [7].

SUMMARY

The behavior of liver enzymes involved in G-6-P utilization (glucose-6-phosphatase, phosphoglucomutase, and phosphohexoseisomerase) was examined during 6 days of fasting. Liver/body weight ratio, liver weight, cellularity, and nitrogen content of the homogenate and supernatant fluid were also determined. The enzymatic activities were expressed per unit liver (per wet weight, per average cell) and per 100 g body weight (total available enzymatic activity).

1. The 6-day fasting caused a progressive decrease in liver/body weight ratio and in liver weight. On the other hand, the cellularity progressively increased. The total cellularity, however, did not change. The nitrogen content of the liver homogenate was apparently increased and that of the supernatant fluid did not change during the 6-day fasting period when values were expressed on wet weight basis. The real decrease in liver nitrogen content was masked by the crowding of the cells. It was demonstrated that the nitrogen content significantly decreased in the average liver cell, and this change was mostly due to the decrease in the supernatant fluid nitrogen content. The nitrogen content per 100 g body weight also progressively decreased during the fasting period.

2. The alterations in hepatic enzymatic activities were also masked by the shrinkage of the liver during the prolonged fasting period. The hepatic G-6-Pase markedly increased whereas phosphoglucomutase and phosphohexoseisomerase apparently did not change when expressed per wet weight basis. However, when enzymatic activities were expressed per average cell, it was found that G-6-Pase was maintained in the normal range during 6 days of fasting. During the same period phosphohexoseisomerase and phosphoglucomutase progressively decreased and about 70 per cent of their activity was lost by the end of 6 days of fasting. Essentially similar results

were obtained when enzymatic activities were expressed per 100 g body weight.

3. The effect of 6-day fasting on liver enzymes involved in G-6-P utilization was discussed.

This investigation was supported by the Defence Research Board of Canada (Grant 9310-53, Project D 50-93-10-53) and by the Cancer Research Society, Montreal. The valuable technical assistance of Colette Ayotte and Vilma Jansons is gratefully acknowledged.

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BRIEF NOTES

THE EFFECT OF THE ANOMALOUS OSMOTIC COEFFICIENT OF HAEMOGLOBIN ON THE OSMOTIC BEHAVIOUR OF ERYTHROCYTES

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Received March 5, 1958

It is generally recognised that the relation of cell volume to osmotic pressure in the erythrocyte in saline solutions does not conform to the simple Boyle–van't Hoff law. Swelling in hypotonic and shrinkage in hypertonic solutions is always less than that predicted by the law [3, 6]. Ponder [5] used the following equation to express the Boyle–van't Hoff law:

$$V = W(1/T - 1) + 100, \tag{1}$$

where V = total cell volume; W = volume of water in the cell at isotonic osmotic pressure; and T = tonicity (i.e. relative osmotic pressure) of immersion medium (isotonic osmotic pressure = 1).

The deviation from ideal behaviour could be expressed by multiplying the actual isotonic water content, W, by an empirical factor, R (represented here by a Gothic $\mathfrak{R}$ to distinguish it from the gas constant), before insertion into the equation, thus obtaining the apparent isotonic water content, $\mathfrak{R}W$. Eq. (1) then becomes

$$V = \mathfrak{R}W\,(1/T - 1) + 100. \tag{2}$$

Since the form of Ponder's equation an the symbols used do not conform to the conventional equation used by Lucké and McCutcheon [4] in their classic review or to standard thermodynamic terminology, it has been found convenient to use different but equivalent symbols and equations; cf. [3]. Ponder's equation (2) is a statement of the simple and approximate form of the Boyle–van't Hoff law and is exactly equivalent to Lucké and McCutcheon's equation

$$\Pi(V - b) \simeq K = \Pi_0 (V_0 - b) \simeq W_0, \tag{3}$$

where Π ($\equiv T$) = osmotic pressure
Π_0 = isotonic osmotic pressure (taken as unity)
V_0 = isotonic total cell volume
b = non-solvent volume
K = a constant
W_0 ($\equiv \mathfrak{R}W$) = apparent isotonic volume of cell water $\simeq V_0 - b$.

If eq. (1) is differentiated (assuming the accuracy of the approximate equalities) there results

$$\frac{dV}{d(1/\Pi)} = K = W_0. \tag{4}$$

In this terminology, Ponder's $\mathfrak{R} = W_0/W_m$, where W_m ($\equiv W$) is the true isotonic volume of cell water.

The value of Ponder's $\mathfrak{R}$ depends on the physiological state of the erythrocyte and on the anti-coagulant used [6]. However, the value in the physiologically normal erythrocyte of defibrinated or heparinised blood has been found to lie in the range 0.9–1.0; an average experimental value of 0.95 was obtained by Dick and Lowenstein [3]. This deviation of $\mathfrak{R}$ from 1.0 was shown to be due to the large osmotic coefficient of haemoglobin at the high concentration present in the corpuscle. On this basis the value of $\mathfrak{R}$ was predicted by numerical calculation from known physico-chemical data on the erythrocyte constituents, particularly the osmotic coefficient of haemoglobin [3]. It has now become possible to predict the value of $\mathfrak{R}$ by a much shorter and simpler calculation.

Eq. (3) is an approximation which depends on the assumption that the osmotic coefficients of all fractions of the cell solute are constant within the range of concentrations produced in osmotic experiments. The exact form of the Boyle-van't Hoff law is

$$\Pi (V - b) = \varphi RTn, \tag{5}$$

where φ = average molal osmotic coefficient of intracellular solute
R = gas constant
T = absolute temperature (distinguish from tonicity in Ponder's equation)
n = number of gram-molecules of solute in volume V.

Eq. (3) is only true if φRTn is a constant, that is, if φ is a constant, since R, T, and n are kept constant during osmotic experiments. The differentiated form of eq. (3)

$$\frac{dV}{d(1/\Pi)} = W_0$$

also depends on this assumption.

It is known from the data of Adair [1] that the osmotic coefficient of the haemoglobin fraction of the cell solute increases with the concentration, so that φ is in fact variable and therefore W_0 is only the *apparent* isotonic water content of the cell (on the basis of the simple inexact law) and not the actual water content. W_0 is indeed merely an empirical expression of $dV/d(1/\Pi)$. The value of W_0 may be obtained accurately by differentiating eq. (5) correctly, i.e. treating φ as a function of Π (since it depends on the molar concentration of solute). Thus

$$W_0 = \frac{dV}{d(1/\Pi)} = \frac{d(\varphi/\Pi)}{d(1/\Pi)} RTn = \left(\varphi - \Pi \frac{d\varphi}{d\Pi}\right) RTn. \tag{6}$$

On inserting values at the isotonic osmotic pressure, there results

$$W_0 = \varphi_0 RTn - \left(\frac{d\varphi}{d\Pi}\right)_{\Pi=1} RTn \tag{7}$$

(since $\Pi_0 = 1$, and putting $\varphi = \varphi_0$ at isotonic osmotic pressure).

But W_m, the true isotonic water content of the cell, is actually equal to $(V_0 - b)$, i.e. from eq. (5) on substituting values at the isotonic osmotic pressure

$$W_m = V_0 - b = \frac{\varphi_0 RTn}{\Pi_0} = \varphi_0 RTn \quad \text{(since } \Pi_0 = 1\text{)}. \tag{8}$$

Combining eq. (7) and (8) there results

$$W_0 = W_m - \left(\frac{d\varphi}{d\Pi}\right)_{\Pi=1} RTn. \tag{9}$$

Thus since $d\varphi/d\Pi$ is positive [1], $W_0 < W_m$, and

$$\Re = \frac{W_0}{W_m} = 1 - \left(\frac{d\varphi}{d\Pi}\right)_{\Pi=1} \frac{1}{\varphi_0} < 1.0. \tag{10}$$

Since eq. (10) is derived from fundamental thermodynamic principles it is of general validity for all cells.

The value of $\Re$ may be calculated from eq. (10) by insertion of the appropriate numerical values. $d\varphi/d\Pi$ is evaluated by expanding it as follows:

$$\left(\frac{d\varphi}{d\Pi}\right)_{\Pi=1} = \left(\frac{d\varphi}{dx}\right)_{\Pi=1} \left(\frac{dx}{d\Pi}\right)_{\Pi=1}. \tag{11}$$

From the data of Adair [1] it has been calculated [3] that

$$\varphi_{Hb} = 1 + 1.59x + 4.49x^2 \tag{12}$$

(where x = molal concentration of Hb in cell).

Since at the isotonic osmotic pressure the average normal concentration of haemoglobin in the erythrocyte is 0.33 g/ml of solution, which is equivalent to 0.44 g/ml of solvent (assuming a partial specific volume for haemoglobin of 0.75), then

$$(\varphi_{Hb})_{\Pi=1} = 2.57 \quad \text{and} \quad \left(\frac{d\varphi_{Hb}}{dx}\right)_{\Pi=1} = 1.59 + 8.98x = 5.54.$$

But the average isotonic molal concentration of haemoglobin in the erythrocyte is approximately 6.5 mMol/l solvent (taking the molecular weight of haemoglobin as 66,700) in a total intracellular molal ion and molecular concentration of approximately 300 mMol/l solvent, and the mol-fraction of haemoglobin is thus 0.022. Also

$$\varphi_0 = \frac{\text{Total isotonic osmotic pressure of cell solute}}{\text{Total isotonic molal concentration of cell solute}}$$

$$= \frac{x\varphi_{Hb} + \Pi_c}{x + m_c},$$

(Π_c, φ_c and m_c are the isotonic partial osmotic pressure, molal osmotic coefficient, and molal concentration of the crystalloid fraction of cell solute). But

$$\Pi_c = m_c\varphi_c \doteq m_c \quad (\text{since } \varphi_c \doteq 1.0)$$

and thus

$$\varphi_0 = 1 + \left(\frac{x}{x+m_c}\right)_{\Pi=1}\left[(\varphi_{Hb})_{\Pi=1} - 1\right]$$

$$= 1 + 0.022\left[(\varphi_{Hb})_{\Pi=1} - 1\right] = 1.035. \tag{13}$$

Also from eq. (13)

$$\left(\frac{d\varphi}{dx}\right)_{\Pi=1} = \left(\frac{x}{x+m_c}\right)_{\Pi=1}\left(\frac{d\varphi_{Hb}}{dx}\right)_{\Pi=1} = 0.022\left(\frac{d\varphi_{Hb}}{dx}\right)_{\Pi=1} = 0.122.$$

Since the molal concentration of haemoglobin is inversely proportional to the water content of the cell which in turn is inversely proportional to the external osmotic pressure, i.e.

$$x \propto 1/V \propto \Pi,$$

therefore $(dx/d\Pi)_{\Pi=1}$ = a constant = 0.44 g/ml solvent per unit of isotonic osmotic pressure.

When these numerical values are inserted in eq. (10), then

$$\Re = 1 - \frac{0.122 \times 0.44}{1.035} = 0.95.$$

(If of course values other than the average isotonic haemoglobin concentration were used in the equation, different $\Re$ values would result.) This calculated value is in agreement both with the experimental observations and the results of the previous numerical calculations.

It is a basic assumption of this calculation that the haemoglobin in the erythrocyte is in solution or is at least capable of behaving as if in solution. Although eq. (10) is of general validity for all cells provided this assumption is fulfilled, the discrepancy between the value of $\Re$ predicted by its use and $\Re$ values in chick heart fibroblasts in tissue culture obtained by Dick [2] suggests that this condition may not apply to the protein component in fibroblasts. Further work is needed to settle this point.

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FRACTIONATION OF RAT LIVER PARTICULATES USING POLYVINYLPYRROLIDONE GRADIENTS[1]

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Received March 6, 1958

In earlier experiments on separation of subcellular components of rat liver by gradient differential centrifugation [5, 6], we had prepared gradient tubes with solutions of sucrose varying in concentration from 0.88 *M* at the bottom to 0.29 *M* at the top, above which was layered the tissue homogenate in 0.25 *M* sucrose. The calculation of the distribution of particle sizes associated with a specific enzyme activity was thus complicated not only by gradients of density and viscosity, but also by an osmotic pressure gradient, for which at best a semi-arbitrary correction could be made [5], based on observations by Tedeschi and Harris [4] on the osmotic behaviour of mitochondria in sucrose solutions.

The usefulness of polyvinylpyrrolidone (PVP) in conjunction with sucrose for both biochemical [2] and cytologic [3] studies of cytoplasmic particulates suggested the possibility of varying the concentration of PVP to produce a density gradient without materially altering the osmotic pressure.

Methods.—Gradient tubes were prepared by successive layering of 2-ml portions of 18, 15, 12, 9, and 6 per cent pharmaceutical grade PVP (Plasdone)[2] in 0.25 *M* sucrose, pH 7.3–7.4, into 12-ml tubes of 1.5 cm inside diameter. The tubes were allowed to stand at 1 to 2°C for 72–96 hrs.; after this time there was no evidence of a discontinuity in refractive index, as judged by the method of Anderson [1]. Liver from a fasted female Sprague-Dawley rat was homogenized in 9 volumes of 5 per cent PVP—0.25 *M* sucrose for 15 minutes with a Potter-Elvehjelm homogenizer, 1 ml of homogenate was layered into the gradient tube, and the tube was then centrifuged for varying lengths of time in the swinging rotor of a Lourdes centrifuge operated at 1°C.

Ten successive 1-ml layers were then removed, and enzyme assays were carried out on each of the ten fractions and on the sediment in the bottom of the tube, as well as on the original homogenate.

Particle sizes were estimated as described earlier [6]. The relationship between viscosity and distance from the axis of centrifugation could be adequately described by a binomial, so the integrated equation [6] simplifies to

$$d^2 = \frac{18}{\int \omega^2 t}\left[\frac{\eta_o - \beta L_o + \gamma L_o^2}{\varrho_p - \varrho_o + \alpha L_o}\ln\frac{L_n}{L_o} + \left(\frac{\eta_o - \beta L_o + \gamma L_o^2}{\varrho_p - \varrho_o + \alpha L_o} + \frac{\beta - \gamma L_o}{\alpha} + \frac{\gamma(\varrho_p - \varrho_o)}{\alpha^2}\right)\ln\frac{\varrho_p - \varrho_o}{\varrho_p - \varrho_o - \alpha(L_n - L_o)} - \frac{\gamma}{\alpha}(L_n - L_o)\right],$$

[1] This work was performed under the auspices of the U.S. Atomic Energy Commission.

[2] A generous supply of Plasdone was furnished by the General Aniline and Film Corporation, New York.

where ϱ_0 is the density (1.047 g/cm³) and η_0 the viscosity (0.0747 poise) of the suspending medium at the meniscus, L_0 the distance from the centrifugal axis to the meniscus (6.0 cm), L_n the distance from the axis to the boundary of a given layer, α the coefficient of density change with tube length (0.00587 g/cm⁴), β and γ the coefficients of viscosity change with tube length (− 0.00367 poise/cm and 0.02148 poise/cm², respectively), $\int\omega^2 t$ is the product of the square of the angular velocity and the time (in seconds) integrated over the entire run to account for acceleration and deceleration of the centrifuge, and d the diameter of particles expected to be found at L_n.

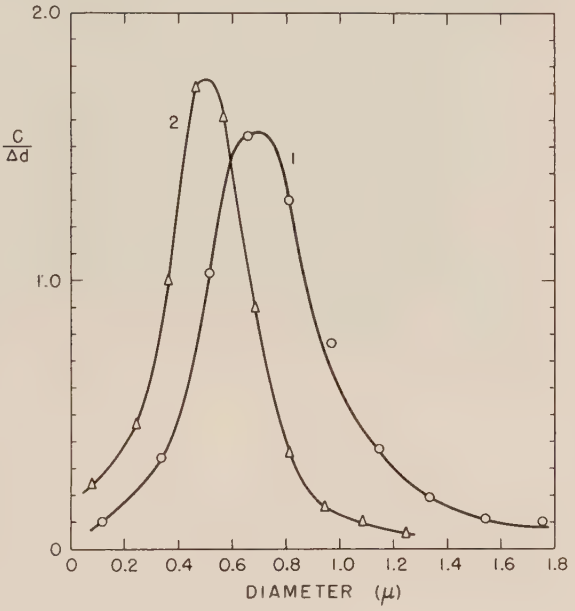

Fig. 1.—Distribution of succinic dehydrogenase (curve 1) and uricase (curve 2) in particulates of rat liver, centrifuged at a speed and time equivalent to 5400 rpm for 1 and 2 hours, respectively, in a sucrose-PVP gradient. The ordinate $C/\Delta d$ is the concentration of activity in a given zone as a fraction of the total divided by the difference between the maximum and minimum particle diameters expected to be found in that zone.

The numerical value for ϱ_p, the particle density, is open to question. In previous work, we had chosen 1.22 g/cm³, obtained from sedimentation equilibrium studies [6], and later we used 1.10 [5], calculated from the data of Tedeschi and Harris [4]. However, the calculation of the latter figure was made with the assumption that mitochondria were not penetrated by sucrose; in view of the recent observations of Werkheiser and Bartley [7] on the permeability of mitochondria to sucrose, 1.10 is probably too low. We have also used gradient tubes containing 4 to 13 per cent PVP in 0.25 *M* sucrose, and have found that the distribution curves obtained with the two different gradients are most nearly superimposable when a value of 1.14 ± 0.01 is used for ϱ_p.

Results.—Fig. 1 shows the distribution of succinic dehydrogenase and uricase in rat liver, calculated with $\varrho_p = 1.14$ g/cm³. At this particle density, the particle size associated with maximum succinic dehydrogenase activity was about 0.05 μ less than that previously reported [5], while the maximum for uricase was about 0.05 μ higher.

This discrepancy is doubtless accounted for by the assumption that the particles containing uricase had the same density and behaved the same osmotically as mitochondria. It may be noted that the reproducibility of the distribution curves among different rats is about the same with PVP-sucrose as that reported previously for sucrose alone [5].

Discussion.—By keeping the concentration of sucrose constant and varying that of PVP, gradients can be prepared with which centrifugal separation of rat liver particulates can be effected, with results similar to those obtained using sucrose alone. We have also used PVP-sucrose gradients to study enzyme distributions in rat thymus. Changes in particle size due to osmotic effects, as well as possible changes in function, are negligible, and the PVP-sucrose medium is excellent for electron microscopy [3]. Among the disadvantages of the use of PVP are the greatly increased viscosity of the medium, so that greater centrifugal forces must be employed; the interference with protein N determinations [2]; and the necessity of homogenizing for longer periods of time to disrupt the cells [2].

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PROTEIN SYNTHESIS IN VACUOLATED RAT LIVER CELLS

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Received March 9, 1958

Biochemical studies on the vacuolar degeneration of rat liver cells have been carried out in this Institute during the past four years.

Vacuolated liver slices have a normal QO_2 and retain their ability to oxidize octanoate and succinate, but their R.Q. is significantly lowered [1]. In spite of the fact that oxidative phosphorylations *in vitro* are not impaired in isolated mitochondria [3], the analysis of phosphorylated intermediates *in vivo* has demonstrated that labile P (largely ATP-ADP) and total high energy phosphate bonds have decreased in vacuolated liver [4]. Protein catabolism does not seem to be affected in vacuolated liver cells, as no modification has been found in nitrogen fractions: catheptic activity and acid-soluble –SH groups are unchanged [4].

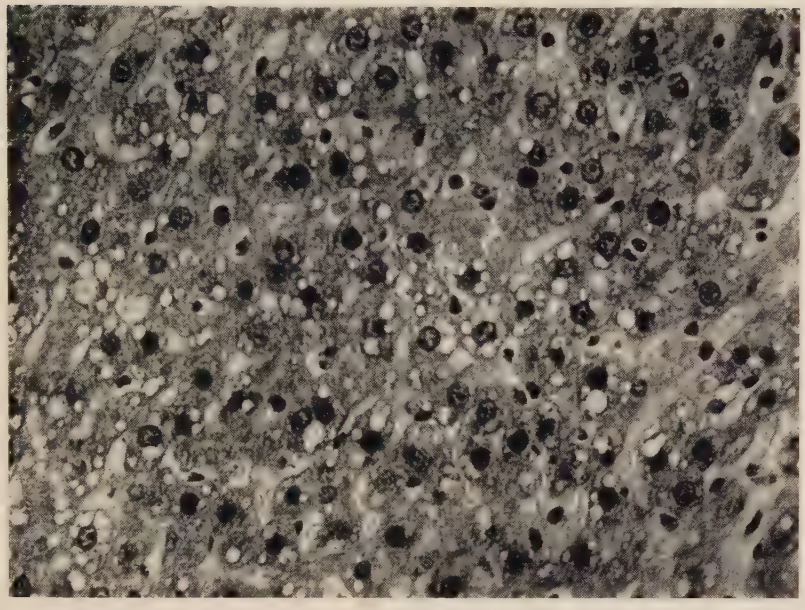

Fig. 1.—Vacuolated rat liver cells. Hematoxylin-eosin staining, ×440. The vacuoles do not contain fat or glycogen.

The present investigation deals with the problem of protein synthesis in vacuolated liver cells *in vitro*. We have studied the incorporation of various labeled amino acids into liver proteins and the synthesis of *p*-aminohippuric acid (PAH) from *p*-aminobenzoic acid (PAB) and glycine, which represents a convenient model for the study of peptide bond synthesis [9]. The release of nitrogen from incubated liver slices has also been investigated.

Experimental.—16–18 hrs fasted albino rats, of either sex, weighing between 200 and 250 g were used throughout our experiments. Liver cell vacuolation (Fig. 1) was obtained according to Pichotka's technique [17] by keeping the animals in a continuously renewed atmosphere of 3 per cent oxygen and 97 per cent nitrogen for 2 hrs. At the end of the treatment, the rats were stunned and exsanguinated, the livers quickly removed and sliced with a free-hand razor; a small part of liver was taken for histological examination. The same procedure was adopted with normal rats.

Incorporation experiments. The slices were then placed into the main chamber of a Warburg flask containing 3.2 ml of Ringer-phosphate incubation medium (final concentrations: NaCl, 1.28×10^{-1} *M*; KCl, 1.35×10^{-2} *M*; $CaCl_2$, 1.94×10^{-3} *M*; $MgSO_4$, 6.8×10^{-4} *M*; phosphate buffer pH 7.2, 1×10^{-2} *M*).

The side arm contained 2 μc of the different labeled amino acids (referred to the L-form) and a certain amount of the unlabeled compounds up to 3.4 μM (referred to L-form) in a total volume of 0.2 ml. The amino acids (glycine-1-^{14}C, DL-leucine-1-^{14}C,

DL-3-phenyl-alanine-2-^{14}C) were always dissolved in distilled water. 0.2 ml of 20 per cent KOH were placed in the center well. The incubation was carried out at 38°C in air; and flasks were shaken about 100 times per min. After a 10 min. equilibration period the labeling reaction was started by transferring the amino acids from the side arm into the incubation medium: that was within 30 min. of the death of the animal.

The labeling reaction has been carried out for 1 hr, as preliminary experiments had shown that for both control and treated rats incorporation is linear during at least 80 min. incubation [12]. After the incubation the flasks were withdrawn from the bath and placed in cracked ice: the slices were removed, washed in chilled distilled water, gently blotted on filter paper and homogenized in 5 ml of distilled water; 1 ml of 6 per cent trichloroacetic acid (TCA) was finally added and the precipitate washed and extracted according to Rabinovitz *et al.* [18].

The dried protein was suspended in absolute ethanol (0.75 ml/mg of protein) using a Potter–Elvehjem all-glass homogenizer. 0.3 ml of the suspension (0.4 mg of protein) were poured into 2 cm^2 copper planchettes and dried. The samples made in duplicate had a uniform thickness and were suitable for measurement. They were counted by using a thin mica-window G.M. counter (type EW 3H, 20th Century Electronics), attaining a precision of 3 per cent in each counting.

Release of nitrogen. Approximately 25–30 mg (dry weight) of rat liver slices were placed in Warburg flasks, containing 3 ml of Ringer-phosphate incubation medium (see above). Kline's [15] procedure was followed; incubation was carried out at 38°C; gas phase air; shaking, 100 strokes/min.

Flasks were removed at the end of 15 min. and 3 hrs and 15 min. Liver slices were removed, gently blotted on filter paper and dried to constant weight. Appropriate aliquots were taken from the supernatant for protein-N determination according to the method of Lowry *et al.* [16] and for amino-N analysis according to Frame, Russell and Wilhelmi [11].

p-Aminohippurate synthesis. PAH synthesis was studied according to the method of Cohen and McGilvery [8]; approximately 25–30 mg (dry weight) of rat liver slices were used, suspended in Ringer-phosphate (see above) containing 40 μM of glycine (final concentration: 1×10^{-2} M) and 6 μM of sodium *p*-aminobenzoate (final concentration: 1.5×10^{-3} M). After 4 hrs incubation in Warburg flasks at 38°C (gas phase, air; shaking, 100 strokes/min.), the slices were removed and dried to constant weight: PAH was estimated in the supernatant according to the technique of Bratton and Marshall [5] as modified by Cohen and McGilvery [8].

Results and discussion. —The effects of cell vacuolation upon amino acid incorporation into the proteins of rat liver slices are shown in Table I.

Three different amino acids have been tested; the rate of incorporation is always significantly lowered in vacuolated livers (glycine, $P < 0.05$; leucine, $P < 0.01$; phenylalanine, $P < 0.05$).

As it seems clear that amino acid incorporation corresponds to a real protein synthesis [19] it may be stated that protein synthesis is impaired in vacuolated liver cells.

The release of protein nitrogen from the slices into the medium is not significantly different ($P > 0.9$) in normal and vacuolated liver slices and the same is true for amino nitrogen ($P > 0.4$) (Table II). Therefore it seems clear that the difference in

TABLE I. *Incorporation of amino acids*[a] *into protein of normal and vacuolated rat liver slices.*

Mean ± S.E.H.; number of experiments in parentheses.

	Glycine	Leucine	Phenylalanine
Normal	3.13 ± 0.25 (8)	2.74 ± 0.02 (8)	1.85 ± 0.05 (8)
Vacuolated	2.45 ± 0.14 (8)	1.41 ± 0.04 (8)	1.36 ± 0.01 (8)

[a] μM per g per hr.

TABLE II. *Release of protein- and amino-nitrogen from normal and vacuolated liver slices incubated at 38 °C in Ringer-phosphate medium, during interval from 15 to 195 min.*

Mean ± S.E.M; number of experiments in parentheses.

	Protein–N (μg/mg dry weight)	Amino–N (μg/mg/dry weight)
Normal	9.96 ± 0.75 (8)	1.54 ± 0.12 (8)
Vacuolated	10.03 ± 0.72 (8)	1.64 ± 0.08 (8)

TABLE III. *Synthesis of PAH by normal and vacuolated rat liver slices incubated at 38 °C in Ringer-phosphate medium.*

Mean ± S.E.M,; number of experiments in parentheses.

	μg PAH formed/mg dry weight/4 hrs
Normal	14.00 ± 0.71 (8)
Vacuolated	9.20 ± 0.43 (8)

amino acid incorporation cannot be explained on the basis of a different catabolic rate.

Further stress is laid on the impairment of synthetic activities in vacuolated rat liver cells by the results shown in Table III.

The synthesis of PAH from PAB and glycine by rat liver slices is also lowered in vacuolated cells. This is in agreement with previous findings on hypoxic rats, which showed an impaired acetylation of aromatic amines *in vivo* which also involves the synthesis of a CO · NH bond [2].

The data presented here indicate that in vacuolated liver cells occurs an impairment in synthetic activities.

It is known that amino acid incorporation into proteins and PAH synthesis are endergonic reactions and the need for coupling with energy-rich phosphate systems has been emphasized [7, 13, 14]. Therefore it would be tempting to correlate the synthetic failure with the decreased amount of labile P in vacuolated liver cells. However, first of all it would be necessary to make clear why isolated mitochondria from vacuolated cells retain their normal phosphorylating capacities [3]. The hypothesis we have put forward, i.e. that uncoupling conditions exist only in the living cells, has not been demonstrated by now.

Moreover experiments carried out in this department have shown that amino acid incorporation into proteins is enhanced in cloudy swelling [6] and in liver regeneration following subtotal hepatectomy [13]; in both cases the oxidative phosphorylations are clearly uncoupled [10, 7]. That seems to demonstrate that the dependence of amino acid incorporation on oxidative phosphorylations is more complex, and that different exergonic reactions may also play a significant role.

Experiments with properly fortified homogenates and isolated cell particles are now in progress, in order to obtain further insight into the problem of protein synthesis in vacuolated liver cells.

Summary.—The incorporation of ^{14}C-labeled glycine, leucine and phenylalanine into liver proteins has been studied on slices from normal and vacuolated rat liver.

Vacuolated liver cells incorporate these amino acids at a rate significantly lowered as compared with normal controls. The release of protein-N and amino-N from the slices into the incubation medium is the same in normal and vacuolated cells.

The synthesis of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine, which has been chosen as an example of peptide bond synthesis, is impaired in vacuolated liver cells.

The possible relationship of these observations to protein synthesis and general metabolism of vacuolated liver cells are discussed.

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PLANT LAMPBRUSH CHROMOSOMES[1]

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Received March 10, 1958

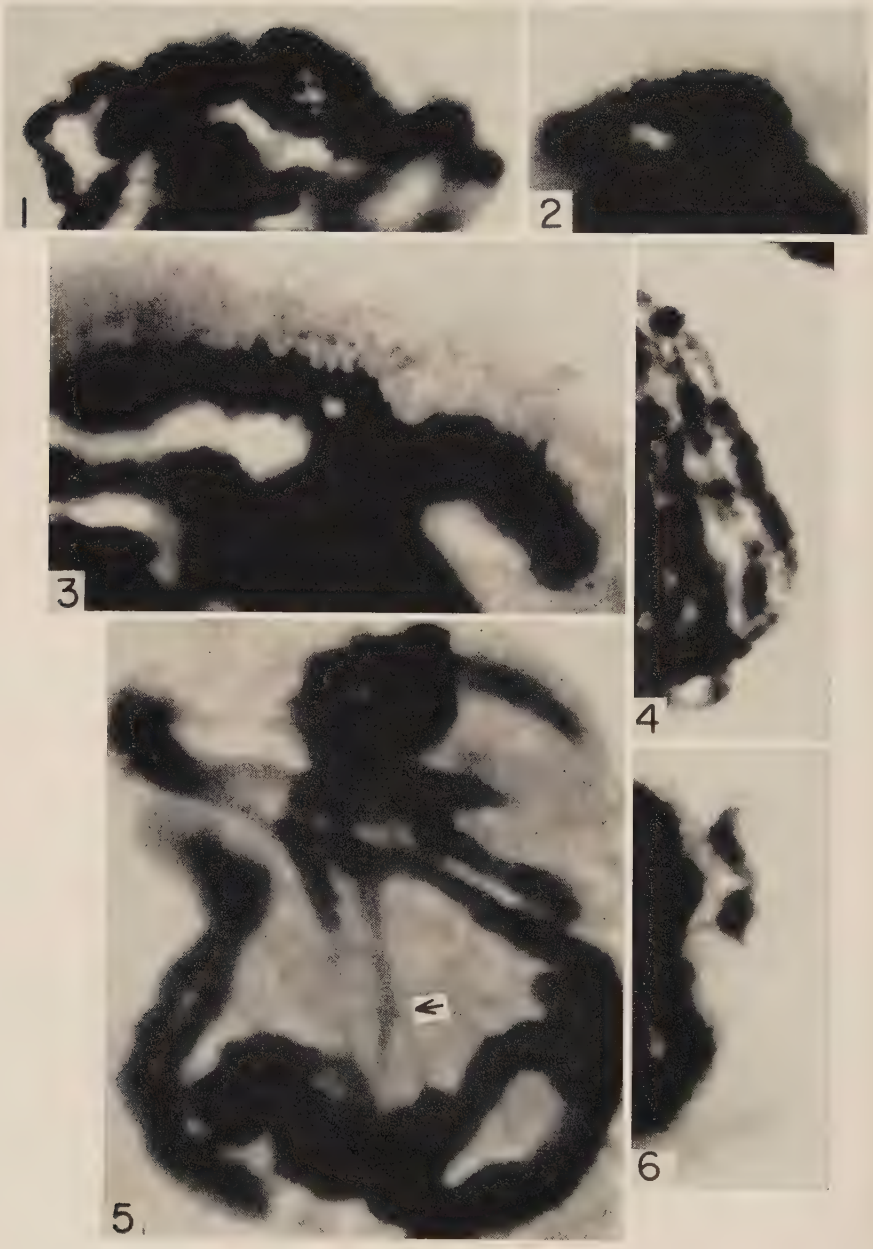
Diplotene chromosomes of oocytes of a number of genera of vertebrate amphibians, birds, and snakes are extremely long, and exhibit a profusion of loop-like processes that have caused them to be named lampbrush chromosomes [1–5, 8]. Similar, but smaller loops have also been seen on diplotene chromosomes of certain insect spermatocytes [6, 7, 10]. It has been assumed generally, however, that these structures are peculiar to the diplotene stage of only certain specific animal and insect groups. The following is a description of lampbrush-type chromosomes found to occur in microsporocytes of a species of nodding onion, *Allium cernuum* Roth.

Materials and Methods.—The plants used were dug from populations native in Centre Co., Pennsylvania, and raised in pots in the greenhouse at University Park, Pa. Buds were fixed during August, 1957 in a Carnoy fixative of 4 chloroform : 3 absolute ethanol : 1 glacial acetic acid. Anthers from buds approximately 2 mm long were smeared in propiono-carmine. Microphotographs were taken of temporary smear preparations and of permanentized preparations [9].

Results.—Some diplotene chromosomes of *Allium cernuum* have processes similar in appearance to those previously described in vertebrate and insect materials. There is a variation within cells in expression of the loop configuration, some chromosome areas merely having an indistinct or fuzzy outline (Figs. 1 and 2) and some having definite, though localized, loop strands (Fig. 3). Intergrades between these extremes, in the form of shorter loops have also been seen. The chromosomes of *Allium cernuum* are not yet well-enough known that specific loop-forming areas, if such exist, can be mapped. These structures are restricted in appearance to diplotene, for none are evident even at late pachytene (Fig. 4) and they have disappeared by diakinesis (Fig. 5).

The strands are extremely thin, and it is therefore impossible to be certain that all are loops. Some appear in Fig. 3 to have terminal knobs, but focussing on such knobs shows that they are artefacts produced by a doubling back of the strands upon themselves to form the loops. Some that cannot easily be interpreted as loops appear to extend from one arm of a bivalent to the other, or even from one bivalent to another. Fig. 6 illustrates such inter-chromosomal diplotene connections between a pair of accessory chromosomes and a bivalent. Strands of this sort are also evident in sporocytes of plants lacking accessory chromosomes. While the normal diplotene loops have disappeared by diakinesis, there are inter-chromosomal connections that exhibit a considerable degree of permanence, appearing at diakinesis (Fig. 5, arrow) and even occasionally persisting to the first metaphase. It is, however, not certain that the

[1] Authorized for publication February 13, 1958, as paper No. 2231 in the Journal Series of the Pennsylvania Agricultural Experiment Station and Contribution No. 228 from the Department of Botany and Plant Pathology, The Pennsylvania State University.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inter-chromosomal connections visible at diakinesis have their origin in the type illustrated for diplotene.

Discussion.—The presence of lampbrush loops on diplotene chromosomes of *Allium cernuum* raises the question of whether they are a general feature of this stage of meiosis. Examination of microsporocytes of *Tradescantia paludosa*, *Zea mays*, *Lilium* sp., and several species of *Solanum* failed to reveal the presence of definite loops, though it was noticeable that diplotene chromosomes frequently have a fuzzy outline such as is illustrated in Figs. 1 and 2. No literature reference to a lampbrush structure occurring in plant material has been found, but the fuzzy appearance has been commonly referred to. This fuzz reflects the fact that the chrosome surface is broken out into small pointed projections that might be interpreted as vestigial loops or as the bases of loops that are too thin to be resolved by the light microscope. If this interpretation is correct it would suggest that the difference between lampbrush and non-lampbrush diplotenes is only one of degree of expression of the same phenomenon.

The difference between the loop structures and the inter-chromosomal strands is not clear. Such structures also appear in insect material as seen, for example, in illustrations of the American cockroach [6]. The connecting strands may arise through a sticking together of loops from neighboring chromosomes. Their persistence during diakinesis might be accounted for if one assumed that the normal loops disappear by coiling back into the main chromosome body, for connections between strands from different chromosomes could effectively stop such a coil-back process.

Summary.—Loop processes similar to those previously described on lampbrush chromosomes occur on some regions of diplotene chromosomes of *Allium cernuum*, the nodding onion. Some strands also appear to connect chromosome arms. The loops disappear by diakinesis, but connecting strands sometimes persist. The difference between lampbrush diplotenes and the normally fuzzy non-lampbrush diplotenes may be interpreted as one only of degree of expression of the same phenomenon.

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Note.—All figures are reproduced at ca. 2000 ×.
Figs. 1 and 2.—Portion of a diplotene nucleus showing fuzzy chromosome outlines.
Fig. 3.—Portion of a diplotene nucleus showing appearance of loop-strands.
Fig. 4.—Portion of a pachytene nucleus. Lampbrush strands are not generally visible at this stage.
Fig. 5.—Portion of a diakinesis nucleus. Lampbrush strands have disappeared by this stage. The arrow points to a typical inter-chromosomal strand.
Fig. 6.—Portion of a diplotene nucleus showing a pair of accessory chromosomes attached to a normal chromosome by inter-chromosomal strands.

DEPOSITION OF WALL MATERIAL IN THICKENED PRIMARY WALLS OF ELONGATING PLANT CELLS[1]

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Received March 10, 1958

Rapidly elongating plant cells such as collenchyma and epidermal cells often have thickned primary walls [1, 2, 4]. Studies on the fine structure within such walls have shown that a highly organized arrangement of cellulose microfibrils is maintained throughout the thickness of the wall during the course of extensive elongation. These findings have led to the suggestion that deposition of wall material can occur throughout the thickness of a wall rather than just at the cytoplasmic surface [1, 2, 7]. The present report describes preliminary results of an attempt to test this suggestion directly by applying high-resolution autoradiography, using tritium-labelled sucrose, to thickned primary walls of epidermal cells of *Avena* coleoptiles.

Isolated segments of coleoptiles of *Avena sativa* (Lanark variety) were prepared and grown by the general methods described previously [5, 6]. The segments were floated on a medium containing 0.01 *M* phosphate-citrate buffer (pH 5.0), 8 p.p.m. indoleacetic acid and 2 per cent tritium-labelled sucrose. The labelled sucrose, prepared by the method of Wilzbach [8], was obtained from Merck and Co. (Montreal, Que.) and as received was chromatographically pure and had a specific activity of 40 mc/g. To remove remaining exchangeable tritium, 250 mg of sucrose was three times dissolved in 250 ml of water and evaporated to dryness. Segments, initially 6 mm long, were grown for periods of 3, 9, and 21 hrs on the radioactive solution and attained mean lengths during these times of 7.1, 10.5, and 14.4. mm respectively The segments were killed in 70 per cent ethyl alcohol and half of those from each growth period were extracted for 1 hr in 0.1 *N* HCl at 90°C followed by 1 hr in 17.5 per cent NaOH at room temperature to remove the bulk of cytoplasm and non-cellulosic polysaccharides. All segments were then dehydrated and embedded in paraffin. Transverse sections of the segments, 5 6 microns thick, were mounted on slides, deparaffined in xylol and passed through alcohol to water. Autoradiographs were prepared by the stripping film technique using Kodak (London) A.R. 10 plates. They were exposed for 10 days and developed and observed as described previously [6].

Because of the very low energy of the beta radiation from tritium the resolution of the autoradiographs was better than 1 micron, as judged by the lack of scattering of the silver grains from the edges of the walls. Since the outer walls of epidermal cells of *Avena* coleoptiles are 2–4 microns thick it was possible to determine whether deposition occurred throughout the wall or only on the inside.

Outer walls of epidermal cells from unextracted segments grown for 3 hrs in radioactive sugar showed no measurable uptake of isotope while the cytoplasm (*Cy*) at their inner surface was noticeably radioactive (Fig. 1). Walls from unextracted seg-

[1] Issued as National Research Council paper.

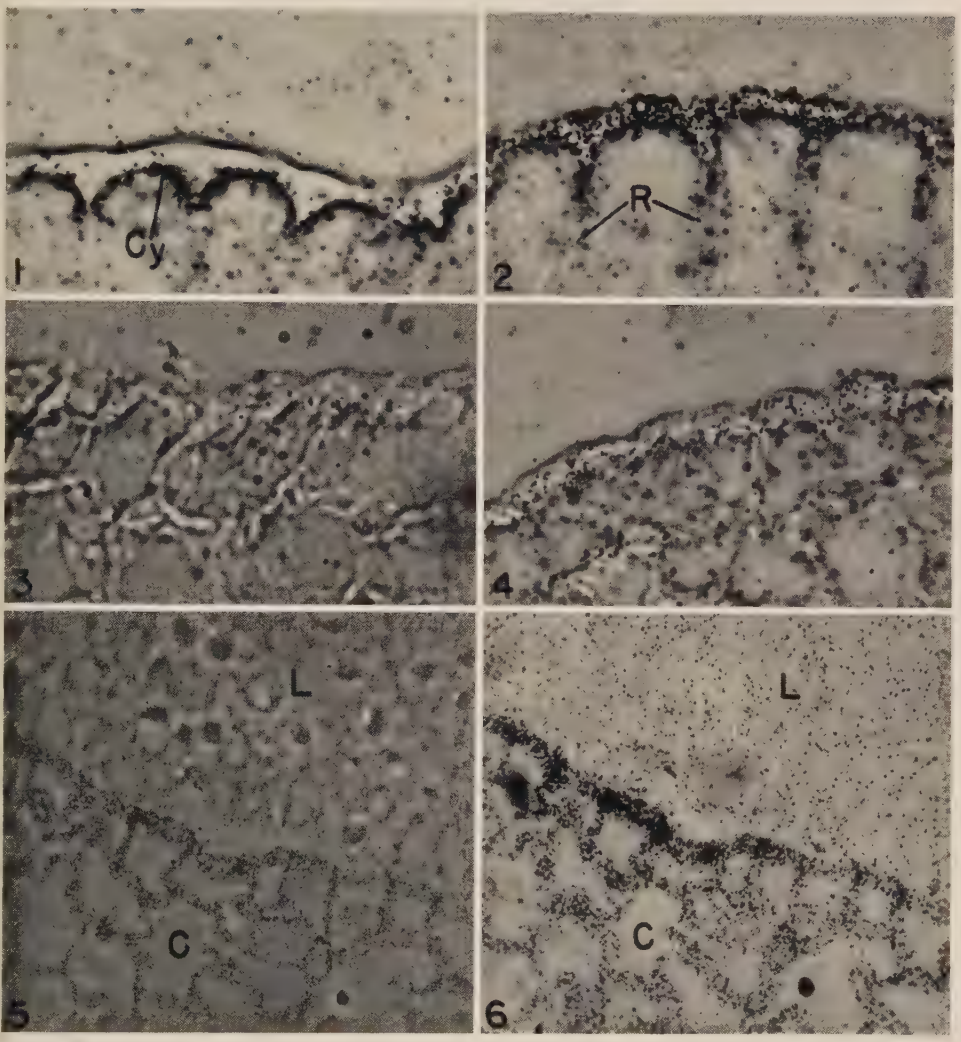

Fig. 1.—Photomicrograph of a transverse section of the outer epidermal walls, with autoradiograph, of cells in an unextracted coleoptile segment grown for 3 hrs on tritium-labelled sucrose. *Cy*, cytoplasm. × 900.

Fig. 2.—Same as Fig. 1, except the segment was grown for 21 hrs on tritium-labelled sucrose. *R*, flattened radial walls. × 900.

Fig. 3.—Same as Fig. 1, except the segment was extracted with HCl and NaOH. × 900.

Fig. 4.—Same as Fig. 2, except the segment was extracted with HCl and NaOH. × 900.

Fig. 5.—Phase-contrast photomicrograph showing both leaf (*L*) and coleoptile (*C*) tissue. From an extracted segment grown 21 hrs on tritium-labelled sucrose. × 360.

Fig. 6.—Bright-field photomicrograph of the autoradiograph of the section shown in Fig. 5. × 360.

ments grown for 21 hrs in radioactive sugar, on the other hand, showed the presence of isotope throughout the entire thickness of the wall (Fig. 2). In all preparations drying of the autoradiographic film caused the thin radial walls of epidermal cells (e.g. *R*, Fig. 2) and parenchyma walls to fall over so that they were at a different focal plane from the outer epidermal walls. Unextracted epidermal walls from segments grown 9 hrs in radioactive sugar also showed the presence of isotope throughout their entire thickness although the frequency of silver grains in the autoradiograph was lower than in Fig. 2.

Epidermal walls from extracted segments grown for 3 hrs in radioactive sugar showed little or no autoradiograph (Fig. 3). No visible cytoplasm remained in these cells. In Fig. 3 the outer walls have been flattened by the film during drying. Walls from extracted segments grown for 21 hrs in radioactive sugar showed the presence of isotope throughout their thickness (Fig. 4) although the density of the autoradiographs was considerably less than that from comparable unextracted walls (Fig. 2). Extracted walls from segments grown for 9 hrs in radioactive sugar gave a very light autoradiograph with grains distributed over the entire wall thickness.

Several possibilities which might produce artifacts invalidating the results should be considered. Physical or chemical reaction between walls and emulsion, adsorbed sugar, or the presence of exchangeable tritium atoms in the radioactive sugar could produce an autoradiograph unrelated to synthesis of new wall material. Fortunately the primary leaves within the coleoptile segments serve as a control in the experiments. These leaves elongated only very slightly and would be expected to show very little metabolic incoporation of labelled sugar as a result of synthesis of new wall. Figs. 5 and 6 show that the walls of the leaf cells (*L*) did indeed give only a very weak autoradiograph in comparison to those of the coleoptile cells (*C*). This fact, in conjunction with the finding that the density of autoradiographs over the coleoptile walls increased with increasing time and growth in the presence of radioactive sucrose, indicates that the majority of isotope incorporation in the walls was due to synthesis of new wall material.

The results show that both cellulose and noncellulosic materials are deposited throughout the thickness of elongating outer walls of epidermal cells of *Avena* coleoptiles. This partially explains how the regular multilayered structure throughout the thickness of these walls [1] is maintained during elongation. The detailed mechanism by which deposition of wall material occurs remote from the cytoplasmic surface is unknown. Colvin *et al.* [3] have shown that in cultures of *Acetobacter xylinum* precursor liberated from cells diffuses through the medium and adds to cellulose microfibrils. In thickened cell walls precursors produced by cytoplasm may diffuse through the wall, perhaps partly in plasmodesmata [2], and be polymerized in the wall.

These studies with tritium-labelled sucrose are continuing and will be reported in detail elsewhere.

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THE USE OF THE SAKAGUCHI REACTION FOR THE CYTOCHEMICAL DETERMINATION OF COMBINED ARGININE

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Received March 15, 1958

There is an urgent need for cytochemical methods which can be used in the microphotometric estimation of individual amino acids combined in proteins in the cell. In particular, a reliable method for the study of arginine would be of great value because of the relationship of this basic amino acid with the acidic phosphate groups of the nucleic acids in nucleoprotein complexes [7, 8]. A preliminary investigation [5] has indicated that the Sakaguchi colour reaction might be suitable for the microphotometric estimation of arginine. With the application to microphotometry still in mind, the optimal conditions for the reaction have been determined more precisely and the nature of the coloured reaction products has been investigated. The colour-complexes formed in solutions of arginine, related compounds and proteins have been measured spectrophotometrically and these measurements have been compared with those made on cells in a scanning microdensitometer [3].

Reaction conditions.—The Sakaguchi reaction is a specific test for the guanido group and thus for arginine, since this is the only amino acid containing the group which is known to occur in proteins. The original reaction mixture [6] contained α-naphthol but this can be replaced by several of its derivatives or by some derivatives of quinoline [4, 5]. The relative intensities of the red colour developed by using α-naphthol, 2,4-dichloro-α-naphthol (DCN) and 8-hydroxyquinoline have been determined both in protamine solutions and in calf thymus nuclei. The results obtained *in vitro* and in the cell proved to be closely comparable and in both cases DCN gave the most intense colouration.

In solutions of protamine, thymus nucleoprotein and arginine the light absorption of the colour complex produced by the reaction was found to be directly proportional to the concentration of the solutions. Similar tests have been made, and comparable results obtained, in the cell [5].

The optimal conditions for the reaction with combined arginine have been determined by varying the components of the reaction mixture (Fig. 1). It should be stressed here that there is a difference between the optimal conditions for combined and for free arginine. The optimal conditions for protamine (clupeine) were similar to those for bull sperm and calf thymus nuclei although the reaction had to be carried out in different ways. The protamine solutions were mixed with the reagents whereas the smears of sperm and nuclei to be measured photometrically, were im-

mersed in a mixture of the reagents and later removed when maximum colour had been developed. NaOBr has been shown to give a very intense colour with free arginine solutions under chemical conditions [2], but it cannot be substituted for NaOCl in the cytochemical method. The reason for this is that when the reagents are first mixed, the NaOBr tends to react much more rapidly with the DCN than does NaOCl. The result is that little or no colour is developed in the specimens.

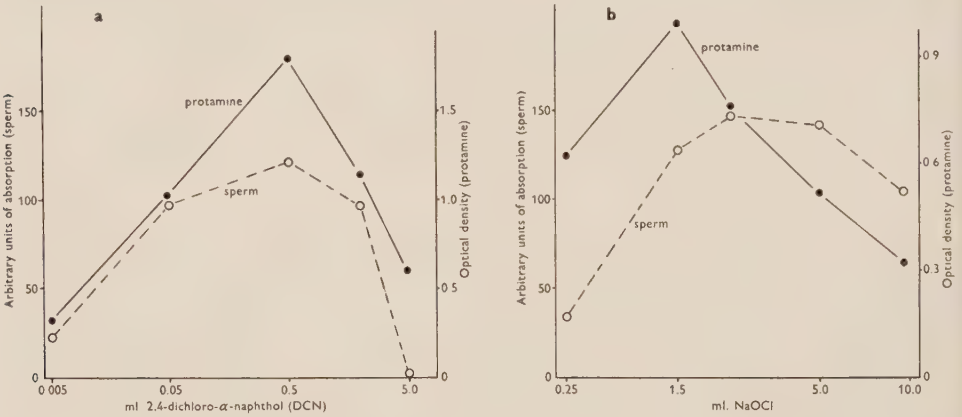

Fig. 1.—Effect of various concentrations, (*a*) of DCN, and (*b*) of NaOCl, on the intensity of colour produced by the Sakaguchi reaction in bull sperm and protamine solutions. (*a*) 1.0 ml 1 per cent DCN with variable amounts of 1.2 per cent NaOCl; (*b*) 2.0 ml 1.2 per cent NaOCl with variable amounts of 1 per cent DCN.

Sperm.—In (*a*) and (*b*) solutions were made up to 50 ml with 1 per cent NaOH. Smears immersed in solution for 6 mins. at 25°C. Each point represents the mean absorption of 10 sperm.

Protamine.—In (*a*) and (*b*) reagents made up to 25 ml with 2 per cent NaOH. 5 ml of reaction mixture was mixed with 5 ml 0.04 per cent clupeine sulphate. After 6 mins. 10 ml of ethanol was added and the absorption measured at 517 mμ.

In all tests reagents were mixed immediately before use. Optimal volumes and concentrations for cytochemical purposes taken as 1.0 ml 1 per cent DCN and 2.0 ml 1.2 per cent NaOCl made up to 50 ml with 1 per cent NaOH. Colour in protamine tends to fade more rapidly than that in smears due to continued exposure to NaOCl.

With protamine solution, using NaOCl and DCN, the reaction produces a red precipitate. A white precipitate is also produced by the addition, to protamine solution, of NaOCl alone. These two precipitates have been analysed for C, H, N and Cl. Their composition, particularly their Cl content, is not very different. This shows that the red reaction product contains fewer chlorinated residues, derived from DCN, than there were guanido groups in the original solution. It is an indication that not all the guanido groups are reacting to give the colour-complex.

Protamine and both the red and white reaction products were hydrolysed by refluxing for 20 hours with 6 *N* HCl. Only the red precipitate formed a small amount of humin-like substance during hydrolysis. The amino acids liberated were then identified by standard techniques of paper chromatography. The seven component amino acids of protamine [1] were detected in each hydrolysate. In the hydrolysates derived from the red and white precipitates an additional compound, glutamic acid, appeared.

Presumably this had been produced by the oxidation of the arginine residues and its formation suggests that there are competing reactions for the arginine residues under the conditions used. The presence of arginine in the hydrolysates provides further evidence that some guanido groups have not reacted.

Spectral measurements.—The visible absorption maxima of the coloured complexes obtained with protamine,[1] thymus nucleoprotein and arginine were measured.

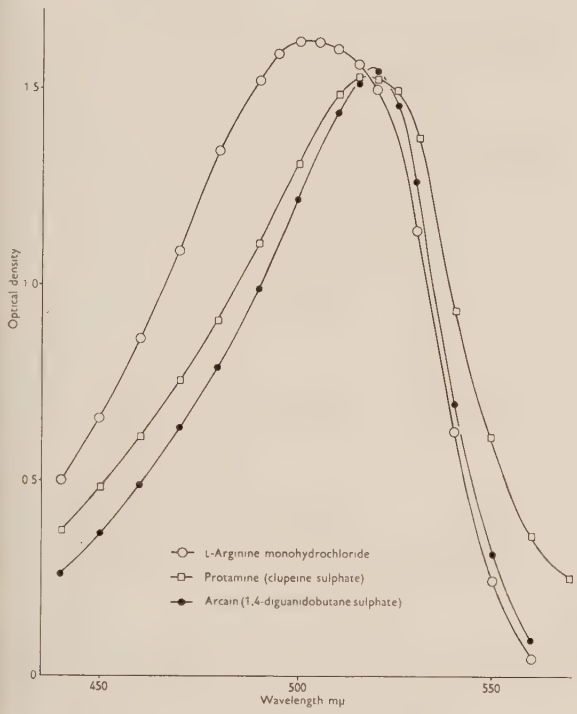

Fig. 2.—Absorption spectra of the red colour-complexes formed by the Sakaguchi reaction in solutions of free arginine, protamine and a simple guanido derivative. An equal volume of ethanol was added to all solutions before measurements were made. Curves for thymus nucleoprotein solutions are similar to those for protamine.

It was found that free arginine gave λ max. at 505 mμ and protamine and thymus nucleoprotein λ max. at 517 mμ. A number of simple guanido derivatives (galegine, arcain, agmatine and streptomycin) and two arginine derivatives (*N*-benzoylarginine and *N*-benzoylarginine amide) gave products with very similar absorption spectra (λ max. 517 mμ) to that given by arginine combined in the protein molecule (clupeine, salmine, thymus nucleoprotein, gelatin, gluten, egg albumin, edestin and casein). These results (see Fig. 2) suggest that the free α-amino and/or α-carboxyl groups of arginine modify the coloured reaction product, or products, and that when these groups are combined in the peptide linkage in proteins, the guanido groups of arginine then react in a similar way to simple guanido derivatives. The colour complexes formed from these derivatives (except streptomycin) are readily extracted into *n*-butanol when their solutions are shaken with this solvent [2], whereas those produced

[1] With protamine the reaction product tended to precipitate in aqueous solution but the addition of one volume of ethanol clarified the solution sufficiently for absorption measurements.

from the proteins are not. This suggests that the colour complex is linked to the protein molecule.

The λ max. of the colour-complex formed with proteins containing arginine is 517 mμ but absorption measurements of cells in the microdensitometer had to be made using the 546 mμ mercury-green line. However, in comparing the reaction in the test tube with that in the cell, this difference has been taken into account.

Application to microphotometry.—If cells are to be measured photometrically for arginine content they must be fixed before the Sakaguchi reaction is carried out. It is essential to know, therefore, which fixatives will give the best preservation and how different fixatives will affect the availability of the arginine for the reaction. So far, 14 different methods of fixation have been tested and the detailed results are to be published later. Briefly, it has been found that the intensity of colour produced by the reaction varies according to the fixative used and to the fixation time. In addition, the standard deviation of single samples of cells tends to be influenced slightly by the fixation. The differences obtained may be due to one, or a combination of, the following factors: to partial loss of protein during fixation; to loss in the reaction mixture; or to differences in the availability of the combined arginine for the reaction.

The results which have been described emphasise the difficulties encountered in attempting to use the Sakaguchi reaction for quantitative cytochemical estimations. By careful attention to conditions, however, the method gives highly reproducible results in any given type of plant or animal cell and it should be possible to use it for comparative purposes provided that the comparisons are not made directly between different species.

Measurements of isolated nuclei from plant root meristems have already been made. The Sakaguchi values have been compared with DNA values obtained from measurements of the amount of Feulgen staining in the same material. In root meristems of *Scilla campanulata* the Feulgen values tended to fall into two classes corresponding with the telophase ($2c$) and prophase ($4c$) amounts of DNA. The Sakaguchi values for combined arginine were also found to fall into these two classes.

Similar measurements have been made on diploid, triploid and tetraploid plants of *Ranunculus ficaria* and on diploid and tetraploid plants of *Tradescantia ohioensis*. In all cases the Sakaguchi values, like the Feulgen values, were found to be directly proportional to ploidy.

While such comparative studies are being continued, with the limitations of the method in mind, further attempts are being made to obtain more information on the effects of fixation and the availability of the arginine for the reaction.

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ELECTRON MICROSCOPY OF CLEAVING SEA URCHIN EGGS

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Received March 24, 1958

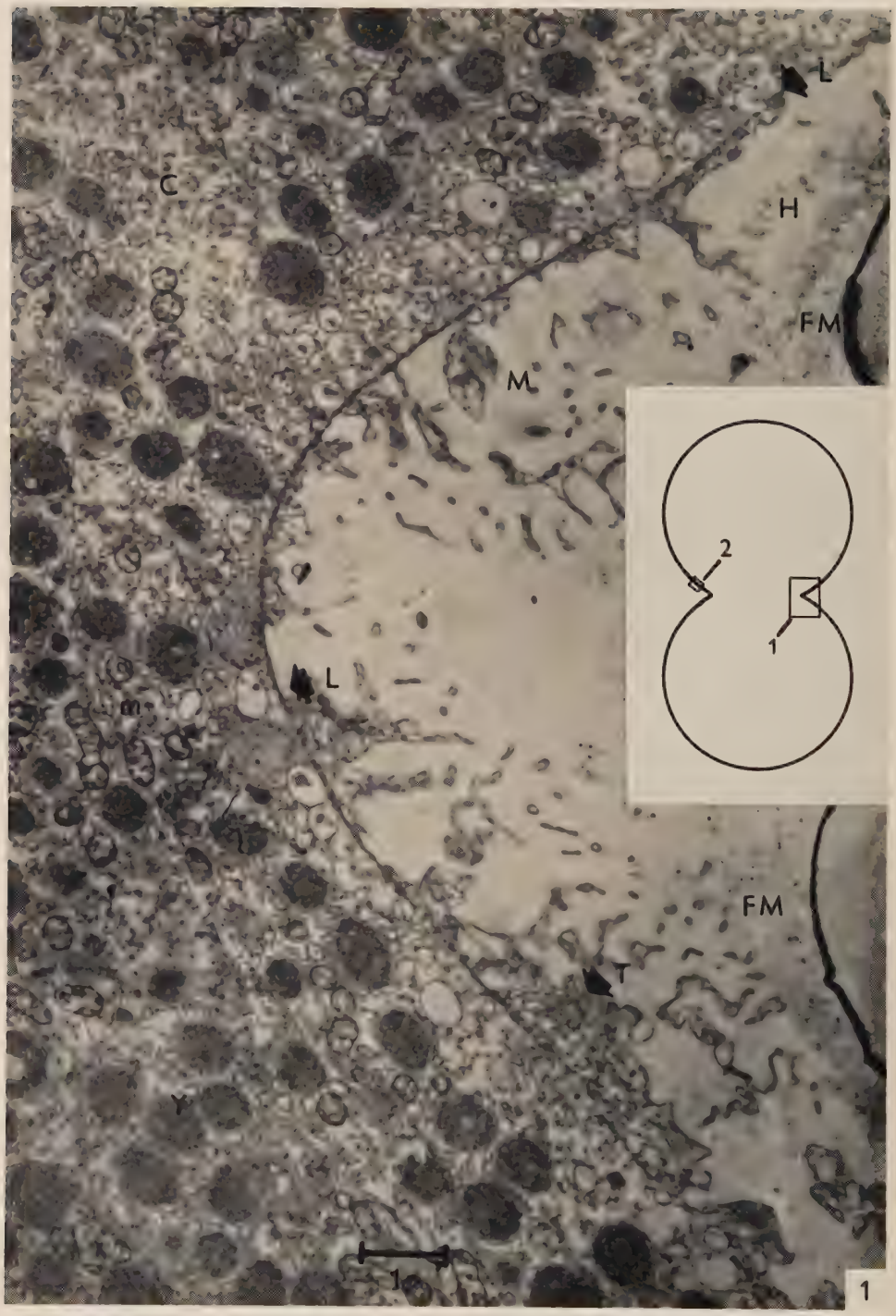
It has been shown that the poles and furrow regions of cleaving sea urchin eggs behave differentially when treated with various chemicals [4] and that the poles are weaker than the furrow [2], but a structural basis for these differences is not apparent in the light microscope [6]. Since the nature of this differentiation is of importance for the understanding of the mechanism of cleavage, we have made a further search by means of electron microscopy.

Methods and observations.—Eggs of the sea urchin, *Psammechinus miliaris*, collected at the Marine Station, Millport, were fixed at a succession of intervals between fertilisation and first cleavage in a standard buffered osmium tetroxide solution (1 per cent OsO_4, pH 7.4 for 2 hours). After dehydration in an alcohol series, they were embedded in an Araldite resin [3]. Ultrathin sections were cut and examined in a Siemens' microscope.

Our observations agree in general with those of Afzelius [1]. The fertilization membrane (*F*, Fig. 1) appears as a thick (ca. 600 Å), dense line splitting into two or more layers. Below this is the wide hyaline space or layer (*H*), containing only small quantities of particulate material. The plasma membrane (*M*) of the egg, which only becomes clearly visible after fertilization, appears as a highly convoluted, thin (~ 100 Å), dense line bounding the densely populated cytoplasm. Rothschild [7] has described this feature and shown that the convoluted nature of the surface is in good agreement with capacitance measurements made on the cell. The cortex, estimated by Mitchison [3] to be of the order of 1 μ thick, is not visible as a distinct structure. This description of the surface layers of the fertilised egg seems to hold until at least 15 minutes before cleavage commences.

At cleavage there appears beneath the plasma membrane an extraordinary profusion of thin tubules (*T*, Fig. 2) which, on occasion, are seen to connect with the membrane and may in fact be derived from it. These tubules run inwards and, turning parallel with the surface of the egg, fuse to form a new, dense layer (*L*, Figs. 1 and 2), which also seems to include a dense, finely textured material. This layer is most strongly developed in the furrow region, as is seen in Fig. 1, and although becoming progressively fainter towards the poles, actually envelopes the entire egg. The sectioned surfaces of the component tubules sometimes suggest that the layer possesses a fibrous texture, but this cannot be asserted.

This new layer forms a smooth continuous curve which follows the shape of the cleaving egg and is never convoluted like the plasma membrane. It is often in close contact with the plasma membrane and, wherever the membrane leaves it to form a convolution (see Fig. 2), tubules are found between the two layers. We have not found any sign of modification of the cytoplasm ahead of the cleavage furrow.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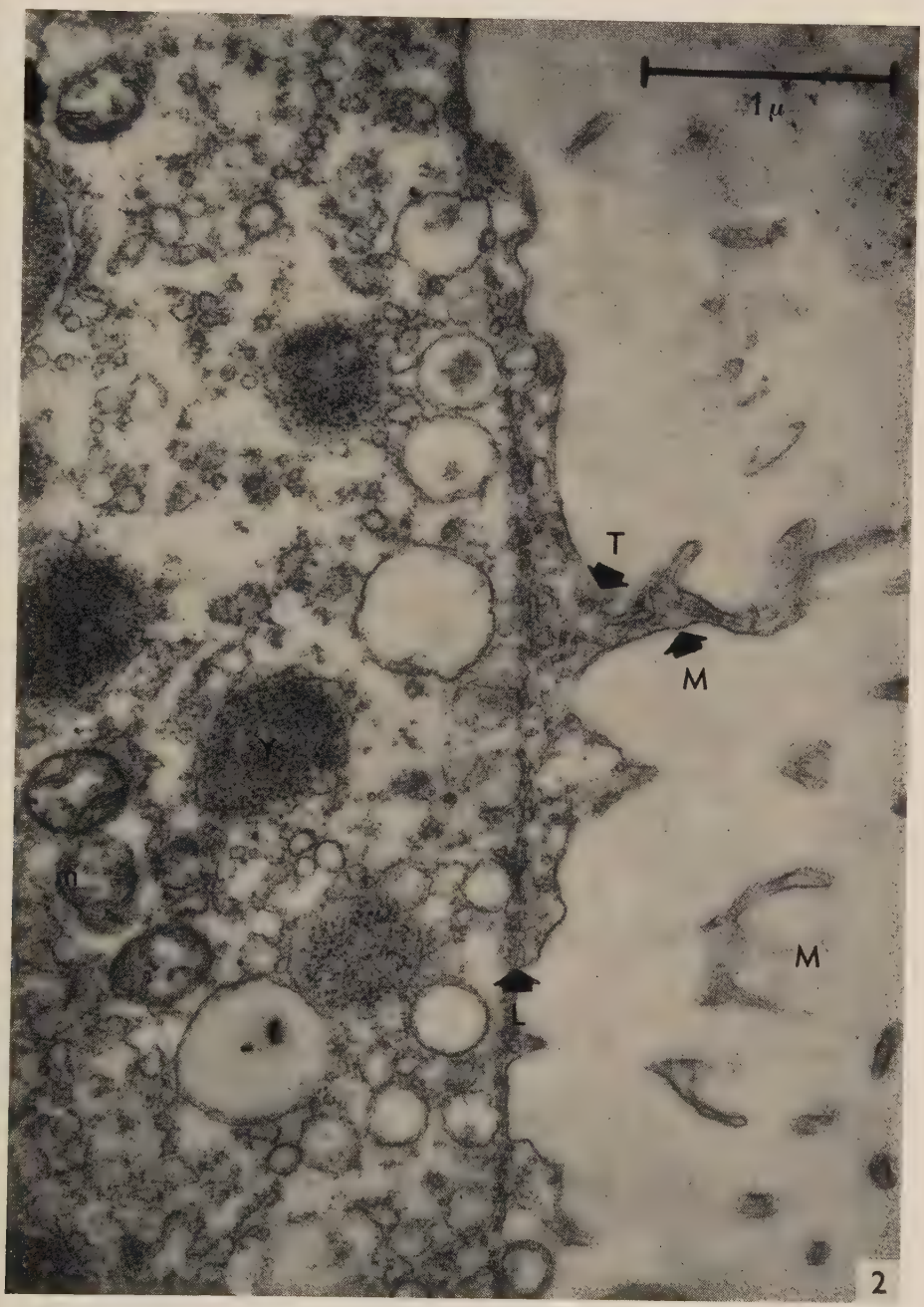

Fig. 1.—Portion of the surface of a cleaving sea urchin egg including the furrow. *M* is the highly convoluted plasma membrane showing protuberances; *FM* is the fertilisation membrane; *H* the hyaline layer; *L* the newly found layer described in the text; *C* the cytoplasm containing yolk granules, *Y*, and mitochrondria, *m*, and a variety of vacuoles, etc. *Inset:* A drawing showing the location of the micrographs relative to the whole egg.

Fig. 2.—An enlargement showing the fine details of the layer, *L*, and the tubules, *T*, between the plasma membrane, *M* and *L*.

Discussion.—The formation of this new layer remains uncertain at this stage of our work, but it seems in part to be derived from the tubules, and the relation between tubules and layer suggests that these structures participate in a peculiar type of sol-gel transformation. The greater density of the layer suggests the presence of heavy atoms derived either from the presence of a new osmophilic component or from the incorporation of such elements as calcium or phosphorus.

The details of its behaviour during cleavage are also obscure. However, its close correlation with the shape of the cleaving egg, and its greater development in the furrow, suggest that it is involved mechanically in the phenomenon of cleavage.

The distribution of this dense material is so similar to that of the contractile gel or ring postulated by Marsland [5] as to lend strong support to his views. A further consideration of this question will be published shortly [8].

Summary.—An electron microscopic study of the cleaving egg of the sea urchin, *Psammechinus miliaris*, shows that a new layer appears below the plasma membrane at cleavage. It is most strongly developed in the furrow and forms a smooth continuous curve following the shape of the cleaving egg. It corresponds closely to the contractile ring postulated by Marsland.

This work was in part supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service. One of us (L. W.) is in receipt of a Nuffield Biological Scholarship.

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THE CONTENTS OF NUCLEOTIDE PYROPHOSPHATES IN AGEING SEA URCHIN SPERMS

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Received March 24, 1958

As is well known, the motility of sea urchin sperms is intensely increased, when a concentrated sperm suspension ("dry sperm") is diluted with sea water [5, 6]. After a short period, however, the initial, violent activity begins to subside ([6], cf. [5, 3, 19, 15, 16, 17]), and a few hours later, at room temperature, the movements are slow and may often give the impression of a certain stiffness of the sperms.

From a functional point of view the locomotive organ of the sperms, the tails, may be compared to primitive muscle fibrils. The comparison may, to a certain extent, be justified also by recent morphological data, obtained by the electron microscope on the systems of filaments in sperm tails and in contractile tissue [2, 1, 12, 10]. It has been shown that adenosine triphosphate (ATP) plays an outstanding role in the contracting muscle fibre, both as a transmitter of energy and as a plasticizer of the contractile actomyosine system. When the ATP-content of a muscle fibre is extensively exhausted, a condition of rigidity develops [18]. A stimulating effect of ATP on the motility of sea urchin sperms has been observed [19]. The present investigation was undertaken in order to decide, whether or not the immobility and seeming stiffness of the sea urchin sperms, which gradually develops after dilution with sea water, is correlated with a progressive exhaustion of the ATP-content.

Experimental.—A concentrated sperm suspension was prepared from carefully rinsed testes of selected specimens of the sea urchin *Brissopsis lyrifera* (Forb.), and was strained through a piece of fine cloth. During the period of collection, which was made as short as possible, the suspension was chilled with ice. Two equal samples were taken from the suspension. The first sample was diluted with 10 volumes of ice-cold sea water and then immediately centrifuged at 0°C. The sediment was repeatedly extracted at 0°C with perchloric acid, as described by Hurlbert *et al.* [9]. The extracts were pooled and neutralized with KOH. The second sample of the sperm suspension was diluted with 10 volumes of sea water at 20°C and was incubated at that temperature for 3 hours. By that time the motility of the sperms had been highly reduced. The sperms were collected by centrifugation in the cold and were then extracted with perchloric acid in the same way as the sperms of the first sample.

The precipitated potassium perchlorate was removed, and the extracts were placed on columns of the anion exchanger Dowex 2 (formate form, 0.13 $cm^2 \times 21$ cm). The chromatograms were eluted with formic acid and ammonium formate of gradually increasing concentrations [9, 8]. The eluates were automatically collected in samples of approximately 1.5 ml. The optical density of the samples was measured at 260 $m\mu$. The nucleotide peaks were preliminarily characterized by the absorption spectra. In the same way as in the previously reported experiments [8] the main nucleotide

TABLE I. *Nucleotide fractions of* Brissopsis *sperm collected from Dowex 2 chromatograms.*

μM of nucleotides per gram of sperm protein.

	Fresh sperms	Sperms aged for 3 hours
ATP	11.0	11.2
ADP	2.5	3.5
AMP	1	1
UTP[a]	5.5	6

[a] Including some GTP.

fractions were further characterized by rechromatography by an ammonium formate system [9]. The fractions obtained were treated with N HCl at 100°C for 60 minutes in order to hydrolyze selectively the purine nucleotides [11, 7]. The reaction products were identified by chromatography on Dowex 50 columns (acetic acid and HCl system, according to Cohn [4]). The identity of the purines was finally confirmed by deamination with sodium nitrite [7] and rechromatography on Dowex 50. The amounts of eluted nucleotides were estimated from the extinctions of the Dowex 2 fractions after subtraction of the corresponding blanks. After a preliminary pilot experiment, two independent experiments of the kind described here were carried out in the spawning season.

Results.—The chromatograms of the acid soluble nucleotides from the *Brissopsis* sperms were characterized by a very dominant ATP fraction. In this respect the acid soluble fraction of the sperms was remarkably similar to that of skeletal muscle, described by Schmitz *et al.* [14]. In the case of the sea urchin sperms, however, the uridine triphosphate fraction was more pronounced. As was reported previously, the uridine triphosphate fraction was fairly large also in sea urchin eggs and embryos [8].

The experiments showed quite unambiguously (Table I) that no significant decrease of the ATP fraction occurred during the incubation period of 3 hours, during which the motility of the sperms was almost entirely lost. The sperms obviously were able to maintain the ATP-content at a high level throughout the period of active motion, very likely by oxidizing endogeneous substrates [3, 6, 13]. The gradually developing motionless state apparently was not due to an exhaustion of ATP, anyhow not in the sperm as a whole.

In the case of the smaller fractions the values obtained by the present method may have only a semi-quantitative significance. In Table I only a few, more prominent of these fractions have been included for comparison.

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X-RAY ABSORPTION STUDIES ON THE NUCLEAR PROTEIN AND RNA CONTENT DURING THE DEVELOPMENT OF THE MITOTIC APPARATUS[1]

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Received March 28, 1958

The mitotic apparatus [11] of the cell is responsible for the regular movements and equal distribution of the chromosomes to both daughter cells. Although the morphology of this cell structure has been extensively studied, the basic mechanism of the formation and function of the spindle and centrosphere is only poorly understood at present. However it is hoped that cytochemical and biochemical investigations on isolated mitotic figures [11, 12] may yield information leading to a better understanding of this essential cell structure. The presence of RNA [11, 1, 15, 17, 19], polysaccharides [13, 18, 20, 10] and SH-group containing and basic proteins [11, 12, 19] in the spindle have been demonstrated. Hypotheses concerning the spindle function have been put forward which are based on cytochemical results [11, 19]. The present study is limited to alterations of the protein and RNA content in the nuclear sap and nucleolus during the onset of nuclear division.

The X-ray absorption technique (historadiography) was employed to calculate the total organic matter of the nuclear sap spindle and nucleolus according to the

[1] This investigation was supported by a grant from the U.S. Atomic Energy Commission (AT-11-1-64 Proj. 8).

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procedure developed by Engström [6, 7] and Clemmons *et al.* [2, 3]. The absorption values were used to calculate concentration of organic matter per μ^3 and the total organic matter was obtained by multiplying the latter data by the volume of nuclear sap or nucleolus respectively. Layers of nitro-cellulose (parlodion) foils were arranged

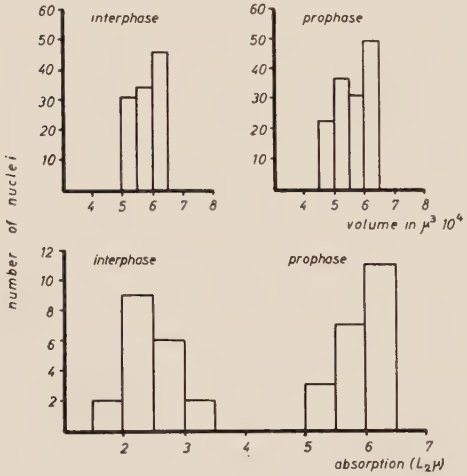

Fig. 1.—Frequency histograms showing the changes in nuclear volume and in concentration of organic matter during interphase and prophase.

in a step wedge in order to provide a reference system for absorption [14]. The following formula was used in calculating the mass per cubic micron (m p/μ^3):

$$m\,\mathrm{p}/\mu^3 = \frac{L_2\mu \cdot B \cdot 1.59 \cdot 10^{-12}}{L_s},$$

where $L_2\mu$ is the absorption value of the object, B is the ratio of absorption constant for the reference material to that of a protein (albumin), 1.59×10^{-12} is the density of the reference material in g/μ^3, and L_s is the thickness of sections (8 μ).

Cytophotometric measurements of the Feulgen reaction were used to determine the DNA content of the interphase and prophase nucleus and of the chromosomes in the metaphase plate. The two-wavelength method of Patau [14] was used, which permits measurement of DNA in irregular objects, such as metaphase chromosomes. The RNA content was estimated by cytophotometric measurements of absorbed gallocyanine-chromalum molecules [5] which provides only an approximation.

The volume of the nuclear sap was obtained by measuring the axes of the nucleus, nucleolus and of the chromosomes in the metaphase plate and subtracting the calculated volume of the two latter structures from the total nuclear volume.

The eggs of *Cyclops strenuus* were used for the study. The large size of the nucleus and mitotic apparatus makes this subject extremely suitable [17, 19]. In addition, the chromosomes are visible in the interphase nucleus. This makes measurement of the nuclear sap possible without any interference from the chromosomes.

The results of the X-ray absorption measurements and of the cytochemical determinations of DNA and RNA are summarized in Fig. 1 and Table I. The amount of

TABLE I. *Alterations of nuclear volume, organic matter (mass), DNA and RNA during the formation of the mitotic apparatus.*

Measurements	Interphase		Antephase		Prophase	Metaphase
	Nuclear sap	Nucleolus	Nuclear sap	Nucleolus	Nuclear sap	Spindle
Volume in μ^3	55,067	4,578	57,114	3,407	56,607	41,088
Organic matter, g $10^{-12}/\mu^3$	0.247	0.568	0.272	0.531	0.418	0.593
Total organic matter in g 10^{-12}/subject	13,601	2,600	15,535	1,809	23,667	24,365
DNA in arbitrary units	5.42		5.25		5.28	5.51
RNA in arbitrary units (semi-quantitattive data)	0		62		85	88

organic substances of the nuclear sap nearly doubles during the prophase. Since the volume of the nuclear sap remains unchanged the concentration of the organic compounds increases. This can also be seen if the frequency histograms on organic matter per μ^3 in interphase and prophase are compared (Fig. 1). The volume of the spindle is around 25 per cent smaller than the volume of the prophase nucleus, thus leading to a further concentration of the organic compounds. The nearly doubled protein content in the prophase raises the question whether we are dealing with a doubling process so commonly found in biological units. In this process only extrachromosomal substances of the nuclear sap could be involved since the chromosomes and nucleoli have been excluded from the measurements. Although the data presented here resemble the doubling processes of the protein content described in spermatocytes [16] and in the uterine mucosa nuclei [8], the results need more evidence for confirmation.

The change in the amount of organic matter in the nuclear sap is mainly due to alterations in the protein content. The lipids, RNA and polysaccharides can be disregarded. The lipids were extracted with an alcohol: ether mixture at 60°C before taking the X-ray pictures. The RNA-amount can be assumed to be less than 4 6 per cent based on the small decrease in X-ray absorption after digestion with RNase. Although these measurements are within the range of technical error and should not be over-emphasized, they are in full agreement with results obtained by Mazia [11]. The amount of polysaccharides incorporated into the nucleus during the late stages of interphase remains constant during pro- and metaphase, as shown by the periodic acid–Schiff reaction [17, 19].

The behavior of RNA seems to be of particular interest in regard to the spindle formation. This substance accumulates in the nuclear sap in the period before the onset of prophase, which is referred to as pre-prophase according to the terminology of Hughes [9]. Following the appearance of RNA, the protein content of the nuclear sap increases. Considering the importance of RNA in the cellular protein synthesis it might be suggested that the accumulation of RNA in the nuclear sap is the basis, if not the cause, of the subsequent increase in protein content. This correlation

between accumulation of RNA and increase in protein content shows that, besides the nucleolus and the chromosomes, a third protein synthesizing system seems to exist in the nuclear sap.

Summarizing the data presented here and that published earlier [17, 19, 18, 20] it can be shown that the spindle formation is preceded by an accumulation of polysaccharides in the nuclear sap during the late interphase, which is followed by the appearance of RNA and a subsequent increase in the concentration of proteins. Only when these synthetic processes are completed the spindle fibers are formed. An interference with these synthetic processes blocks the formation of the mitotic apparatus and consequently nuclear division [20, 21]. It would be worthwhile to consider the possibility that the purine anti-metabolites as well as UV-radiation produce the mitotic inhibition by attacking the RNA-protein synthesizing system leading to the formation of the mitotic apparatus.

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THE EFFECT OF OXYGEN, NITRIC OXIDE, AND RESPIRATORY INHIBITORS ON THE PRODUCTION OF CHROMOSOME ABERRATIONS BY X-RAYS

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Received March 29, 1958

It is now a well-established fact that a given dose of X-rays produces about three times more structural chromosome changes under aerobic than under anaerobic conditions. In root tips of *Vicia faba*, an increase in radiosensitivity becomes evident at about 2 per cent oxygen in the gas phase and at 10 per cent oxygen the sensitivity is usually more than $\frac{3}{4}$ of that in air. Oxygen tensions higher than 21 per cent (air) have no further effect on the frequency of chromosome aberrations [4].

In a recent study of the lethal effect of ionizing radiations on microorganisms, Alper and Howard-Flanders [1, 3] observed a response to oxygen at considerably lower concentrations than those effective in higher plants. When the gas is passed through a suspension of bacteria as a vigorous stream of fine bubbles both during and before irradiation, the oxygen present in ordinary nitrogen cylinders (less than 0.1 per cent) is sufficient to increase the radiosensitivity of bacteria by a factor of about 1.5 over that observed in oxygen-free nitrogen [3]. Howard-Flanders and Alper suggest that the difference between their results with bacteria and the results obtained when organised tissues are used as experimental material may be due to the fact that in their experiment the free oxygen concentration in the cell is more nearly that of the suspending fluid. In organised tissues, such as plant root tips, the oxygen concentration should be much lower in the central part of the root than in the solution surrounding the root because of the respiratory activity of the cells.

In 1956 Lilly and Thoday [6] reported that potassium cyanide (KCN) influenced the frequency of structural chromosome changes produced in *Vicia* root tips by X-rays when the gas phase was nitrogen but not when it was air. Subsequently, the cyanide effect was studied by Kihlman, Merz, and Swanson [5]. It appeared from their studies that with KCN concentrations above 50 μM/l the frequencies of chromosome aberrations in root tips of *Vicia faba* produced by a given dose of X-rays was the same in an atmosphere of nitrogen as in one of air. It also appeared that the ammonium salt of nitrosophenylhydroxylamine (cupferron) had the same effect as cyanide on the chromosome damage caused by X-rays in a nitrogen atmosphere.

The studies on the influence of oxygen and agents reacting with heavy metals on the effect of X-rays have been continued. As before, the experimental material was lateral root tips of the broad bean *Vicia faba*. The radiation consisted of 180 kV X-rays filtered through 1 mm Al and given at a rate of 20 r/min. The root tips were fixed in alcohol–acetic acid, 3:1, 18 hours after the irradiation. Prior to fixation, the roots were treated for 4 hours with 0.05 per cent colchicine. Permanent Feulgen squashes were made as previously described [4]. For each treatment 100 cells from

the root tips of two bean seedlings were analysed for isolocus breaks [8] and exchanges.

Two kinds of experiments were performed. In the first series commercial tank nitrogen (containing about 0.1 per cent oxygen) was introduced into the treatment vessel through a capillary and bubbled through the solution for at least 15 minutes. The vessel was then sealed and irradiated, the entire treatment, including the gassing, taking 0.5 hour. In another series of experiments, the nitrogen was purified by passing it over active copper in a Meyer–Ronge column [7]. According to Meyer and Ronge, nitrogen which has passed through the column at a rate of 10–20 l/hr contains less than 4×10^{-5} per cent oxygen. The gas was introduced into the vessels through a fritted glass filter. In the experiments with cupferron the beans were kept under anaerobic conditions for 10 minutes before an oxygen-free solution of the reagent was introduced. Similarly, in the nitric oxide experiments NO was mixed with oxygen-free nitrogen in an amount determined by a flow-meter after oxygen-free nitrogen had been bubbled through the system for 10 minutes. The vigorous bubbling was continued for 25 minutes, whereupon the beans were irradiated in sealed vessels.

The results of the two series of experiments appear in Tables I and II. Table I shows that KCN and cupferron both considerably enhance the production of chromosome aberrations by X-rays in an atmosphere of tank nitrogen. Hydrogen sulfide (H_2S) and nitric oxide (NO) were also found to be effective. Nitric oxide was tested because Howard-Flanders reported that it has the same influence as oxygen on the radiosensitivity of bacteria [2].

Kihlman, Mertz, and Swanson suggested that the effect of KCN and cupferron on radiosensitivity might be due to a complex formation between these chemicals and heavy metals within the chromosomes [5]. The results of Alper and Howard-Flanders [1, 3] indicate, however, that in plant root tips the oxygen effect may become detectable at considerably lower concentrations of oxygen in the presence of a respiratory inhibitor than in its absence. Therefore, when these results became known, it was evident that the effects of KCN, H_2S and cupferron could very well be due simply to the fact that they increase the intracellular oxygen concentration by inhibiting respiration. KCN and H_2S are strong inhibitors of respiration in plant roots. In a preliminary experiment, 10^{-3} *M* cupferron was found to inhibit bean-root respiration markedly. The irradiations in the presence of the metal reagents had not been performed under completely anaerobic conditions, since unpurified tank nitrogen had been used. Furthermore, since the gas was not introduced through a fritted glass filter, the bubbling might not have been sufficient to remove oxygen. In subsequent experiments, these methodical imperfections were corrected. The experiments could be done satisfactorily only with cupferron, since the vigorous bubbling removed H_2S and HCN as well as oxygen from the solution.

It appears from Table II, that cupferron does not enhance the effect of X-rays under completely anaerobic conditions. One per cent oxygen in the gas phase (14 μM/l in solution) does not influence the radiosensitivity when the inhibitor is absent, but when cupferron is present 1 per cent oxygen has the same effect as 21 per cent on the sensitivity.

These results clearly indicate that the effect of cupferron (and probably also that of KCN and H_2S) on the sensitivity of bean-root chromosomes to radiation is due to its inhibition of respiration. In the absence of respiratory inhibitors, the radiosen-

TABLE I. *The influence of H_2S, cupferron, KCN and NO on the production of chromosome aberrations by 100 r of X-rays in an atmosphere of tank nitrogen (containing 0.1 per cent oxygen).*

Treatment	Abnormal metaphases %	Aberrations per 100 cells Isolocus breaks	Exchanges
Tank N_2	13	9	4
Tank N_2 + 200 μM H_2S	47	39	13
Tank N_2 + 200 μM cupferron	47	44	17
Tank N_2 + 200 μM KCN	42	33	16
Tank N_2 + 20 μM NO[a]	44	34	15
Air (285 μM O_2)[a]	58	52	22

[a] Concentration in the aqueous phase.

TABLE II. *The influence of oxygen, cupferron, and nitric oxide on the production of chromosome aberrations by 100 r of X-rays.*

Treatment	Abnormal metaphases %	Aberrations per 100 cells Isolocus breaks	Exchanges
Oxygen-free N_2	19	17	4
Oxygen-free N_2 + 400 μM cupferron	16	11	5
Oxygen-free N_2 + 30 μM NO[a]	62	37	56
Oxygen-free N_2 + 14 μM O_2[a]	16	13	4
Oxygen-free N_2 + 14 μM O_2[a] and 400 μM cupferron	61	53	28
Air (285 μM O_2)[a]	61	57	30

[a] Concentration in the aqueous phase.

sitivity of the root-tip chromosomes is not affected until the oxygen concentration exceeds 30 μM/l because at lower concentrations the oxygen is removed by respiration before it comes in contact with the sensitive structures. When respiration is inhibited, even low concentrations of oxygen gain access to the nucleus and influence chromosome damage by radiation. Experiments are under way to determine a concentration-effect curve for the influence of oxygen on the production of chromosome aberrations by X-rays in the presence of cupferron.

Table II also shows that nitric oxide appears to be no less effective under anaerobic conditions than when tank nitrogen is used. Although NO, like H_2S and KCN (and probably cupferron) combines with cytochrome oxidase, the enhancing effect of NO

on the production of chromosome aberrations by X-rays apparently is of another kind than is the effect of KCN, H_2S, and cupferron. The fact that nitric oxide readily combines with oxygen also renders it unlikely that its influence on radiosensitivity is dependent on a mechanism which requires the presence of both NO and oxygen. The explanation of the effect of NO given by Howard-Flanders [2], according to which both oxygen and NO influence radiosensitivity by virtue of their affinity for free carbon radicals, is not inconsistent with the results presented here.

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A MUTATION THAT REDUCES NUCLEOLAR NUMBER IN *XENOPUS LAEVIS*[1]

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Received April 12, 1958

The nucleoli in this species are visible under the phase-contrast microscope in a small proportion of the blastula cells; at this time the cells are dividing rapidly and interphase is short. During the course of gastrulation the nucleoli condense somewhat and are seen clearly in most cells not in active division. Organ specific differences in nucleolar morphology become noticeable after gastrulation: those in the central nervous system are smaller than those in the somites, and those in the developing lens which become particularly large are vacuolated. The maximum number of nucleoli in a nucleus depends on the number of chromosome sets present and has been used as an index of ploidy [2]. The majority of non-dividing cells in normal diploids contain two nucleoli, the remainder one, this is so throughout development, but the exact ratio differs from tissue to tissue.

Half the offspring from a single diploid or near diploid female kept in this laboratory possess only one nucleolus in each cell, and these animals fathered by several different males constitute the stock of this new mutation. The viability, growth rate, sex-ratio, and fertility of these heterozygotes do not differ from their wild-type full

[1] Supported by a grant from the British Empire Cancer Campaign.

sibs having two nucleoli; our observations cover the first thirteen months of life and are being continued. Male and female heterozygotes give the same Mendelian ratio as their heterozygous mother when bred to wild type.

The expected Mendelian ratio among the offspring from a cross of two heterozygotes is: one quarter with cells containing two nucleoli, one half having but a single nucleolus in each cell, and one quarter having no nucleoli at all. This ratio has been obtained in our breeding experiments. The development of the homozygous mutants is noticeably retarded after the stage of muscular response, and this class dies as young larvae showing a constant syndrome of abnormalities: they are microcephalic, often oedematous in the heart region, the tail is shortened, its tip crumpled and bent ventrally. These characteristic abnormalities are not limited to this mutation, and very similar syndromes can be realised in a variety of ways, including overcrowding, UV irradiation, and centrifugation.

Squash preparations of the mutant homozygotes covering the embryonic period from the blastula stage onwards have been studied under the phase-contrast microscope. Most nuclei contain bodies having the same phase retarding properties as nucleoli but much smaller in size, although in rare instances they are similar to true nucleoli. In contrast to nucleoli, however, these bodies are usually in a state of Brownian movement, and their boundaries are well defined and lack the fuzziness of the chromatin associated with the nucleolus; they are very seldom seen in preparations of heterozygotes or the wild type where true nucleoli are present. The number of such bodies in a homozygote nucleus can vary between none and eight, but there is some variation from one tissue to another.

Homozygotes in the late embryonic stages have been sectioned and stained with methyl green and pyronin. Nuclei such as those of the central nervous system (which in heterozygotes and wild type contain smaller nucleoli) usually lack pyronin staining inclusions, somite nuclei contain one to four bodies staining but weakly with pyronin. These bodies are much smaller than true nucleoli but exceptions have been observed: the general impression gained from their size and staining properties is that the RNA content of the homozygous nuclei is reduced to less than one eighth of that found in nuclei containing a true nucleolus.

In view of the fact that these bodies have roughly the same density as true nucleoli when observed under the phase-contrast microscope, but have a much lower affinity for pyronin, it is presumed that they are rich in some substance not RNA.

This mutation may be a deletion of the nucleolar organizer region or the whole chromosome that bears it; this will be determined cytologically.

A mutation reducing nucleolar number is potentially useful in two ways. Firstly, it provides an excellent marker visible in interphase nuclei, and we are using it for this purpose in nuclear transplantation experiments [1]. Secondly, it provides a readily available source of material for experiments on the function of the nucleolus in cell metabolism.

A full report with quantitative data and further cytochemical observations will be published shortly.

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THE GLYCOGEN CONTENT OF OVARIAN, BODY CAVITY, AND UTERINE EGGS OF THE FROG, *RANA PIPIENS*[1]

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Received April 15, 1958

Brachet and Needham [4], investigating the glycogen content of the unfertilized frog egg (*Rana fusca*), as well as the developing embryo, indicated there was a continued decrease in the desmoglycogen fraction up to the time of larval hatching. Initially, the ovarian egg had 91.4 per cent of its total glycogen in the free form (lyoglycogen) and as it proceeded through fertilization to hatching this labile component increased to 97.4 per cent. An absolute decrease in total glycogen was observed during this development and the suggestion was made that desmoglycogen preferentially might be transformed into lyoglycogen. A more plausible explanation, according to the authors, is that the egg utilizes the glycogen combined with the proteins (desmo-) in preference to the free glycogen. A mean total glycogen content of ovarian eggs of approximately 1.3 g per cent was reported. In Needham's *Chemical Embryology* [14] a table is presented for the comparison of total glycogen obtained from unfertilized frog eggs by various investigators. Data from three species of *Rana* (*esculenta*, *fusca*, and *temporaria*) are presented [1–2, 5, 7–9] and all but Fauré-Fremiet and duStreel [5] agree essentially on a value of approximately 2 g per cent wet weight. Fauré-Fremiet and another coworker, Dragoiu [6], later obtained a total glycogen value of 3.31 g per cent wet weight.

These [1–2, 4, 6, 9], and other investigators [10, 11], are in general agreement that glycogen is utilized during development of the embryo. Wertheimer [16] claims that approximately one-half of the total body glycogen of the frog is in the eggs and that this "ovarial" glycogen is completely isolated from the body metabolism.

The glycogen values presented in the literature have been determined by a number of different chemical methods and variable results could be expected. Needham [14] inferred this some time ago. Some changes and refinements in technique have been made recently, however, and the work presented in this paper is based upon (*a*) a relatively new technique for the determination of the two glycogen fractions, (*b*) analysis of the glycogen of unfertilized eggs from a different species of frog, and (*c*) analysis of three different types (according to location) of unfertilized eggs namely, ovarian, body cavity, and uterine. The latter were obtained following induced ovulation.

In a previous publication [12], experimental analysis of the acid-soluble (lyo-) and acid insoluble (desmo-) glycogen fractions of the frog oviduct, during the months of December, January, and February, was described. Eggs from these same animals

[1] Supported by Grant #595 from the University of Missouri Research Council, Columbia, Missouri and by Grant #H-2430 (C2), National Institutes of Health, United States Public Health Service.

TABLE I. *Glycogen fractions of frog eggs* (Rana pipiens).

Type of eggs	Glycogen (mg/100 g) Acid-insoluble	Acid-soluble	Total
Ovarian	126±8.0[a]	1941± 80	2067
No. determinations	29	23	
Body cavity	113±6.1	1365±125	1478
No. determinations	5	5	
Uterine	74±5.3	1408± 24	1482
No. determinations	24	21	

[a] Standard error of the mean.

were removed and analysis of the glycogen content is presented in this paper. A sample of these eggs weighing between 200 and 400 mg was obtained and the acid-soluble component determined by a method suggested by Bloom, Lewis, Schumpert, and Shen [3]. The acid-insoluble form was analyzed by a method previously described by Brachet and Needham [4] and modified according to Meyer, Russell, Platner, Purdy, and Westfall [13]. Both fractions were subsequently hydrolyzed with a sulfuric acid-anthrone reagent and the glucose quantitatively measured by spectrophotometric analysis [15].

The experimental results are presented in Table I. Statistical evaluation of the data was determined by simple analysis of variance. The F value of both fractions was slightly over 14 giving a P of $<.001$ in both cases. Application of the t-test, with rejection of the Null hypothesis at the 4 per cent level, indicates there is a significant decrease in the acid-insoluble glycogen of uterine eggs when compared to body cavity and ovarian eggs. No significant difference was noted between the latter two types of unfertilized eggs. In the acid-soluble fraction no statistical difference was observed between body cavity and uterine eggs but both showed a significant decrease from ovarian eggs.

A number of conclusions may be drawn from these data. Total glycogen was obtained by summation of the two individual fractions. This value, for ovarian eggs, is approximately 0.7 g per cent wet weight greater than that obtained by Brachet and Needham [4] in *R. fusca* ovarian eggs. Our value of approximately 2.07 g per cent agrees very well with results reported by previous investigators [2, 7–9]. Brachet and Needham [4] found that 91.4 per cent of the total glycogen of the ovarian eggs was in the acid-soluble or lyoglycogen form. This compares very favorably with the 96.7 per cent obtained in the ovarian eggs of *R. pipiens*. The general statement [4] that "the amount of glycogen combined with protein is less than 10 per cent" is substantiated.

The results also suggest that both glycogen fractions reach their maximum during the period when the eggs are within the ovary. As they are extruded into the body cavity and as they pass through the oviduct and uterus, glycogen is being utilized.

One may therefore speculate that in addition to observing a glycogen decrease from fertilization to hatching [1–2, 4, 6, 9–11], the initial decrease begins at the time the eggs are expelled from the ovaries. In the case of *R. pipiens*, the decrease takes place in both glycogen fractions although the percentage of the acid-soluble form still remains above 90 per cent of the total present. These acid-soluble percentages would appear to form a solid basis for the high values of this fraction reported during initial morphogenesis [4].

One may further conclude from these results that there is a remarkable constancy in total glycogen values presented [2, 7–9] and in fractional glycogen studies [4] even though different methods of glycogen analysis were used. Brachet and Needham determined the acid-soluble glycogen form by the method of Willstätter and Rohdewald [17] which is quite different from the method used [3] in this paper. The absolute lyoglycogen values for ovarian eggs reported by Brachet and Needham are not of the same magnitude as those presented in this paper; however, the relative percentage value for both glycogen fractions is of the same proportion.

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THE DETERMINATION OF DEHYDRATION TIME OF FROZEN-DRIED TISSUES[1]

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Received April 30, 1958

Numerous attempts have been made to determine the drying time of tissues undergoing dehydration by vacuum following rapid freezing. Neither a mathematical approach [11, 16] nor the utilization of pressure measurements, as recorded by a manometer or vacuum gauge [1–4, 6, 7, 10, 13–15], have yielded a sharply definable endpoint. Most operators must learn empirically when drying should be complete by observing that bubbling does not occur when the specimen is embedded in molten wax *in vacuo* and/or by microscopic examination. Recently, Hopkins [8, 9] outlined a simple method that was used for estimating the dryness of intact frog muscle. It was noted that periodic warming of a liquid nitrogen trap located near the tissue produced a marked rise in pressure as a result of the release of sublimed water to a more distant trapping surface. When this intermittent warming of the cold finger no longer resulted in a pressure rise, the tissue was found to be dry, as indicated by the stability of the mechanism which was responsible for rehydration contraction. To determine the accuracy and effectiveness of this method for tissues which were to be embedded and prepared for microscopic examination, the present investigation was undertaken.

Methods.—The freeze-drying apparatus followed the basic design of Glick and Malmström [5], except that a side arm was added to the tissue holder portion for carrying in thermocouple wires (Fig. 1). An ionization gauge tube (Type VG-1A) was located between the two trapping surfaces and connected to either an Ionization Gauge, Type DPM-36 or Type DPA-38 (Consolidated Vacuum Corp., Rochester, N.Y.). Vacuum was obtained by a Duo-Seal two-stage mechanical pump (Model No. 1405B) having a rated capacity of 58 liters/min. For measuring the temperature of the tissue during dehydration, a 30 gauge copper-constant thermocouple was placed within one of the tissue samples. The thermocouple leads were connected to a galvanometer or to a direct reading pyrometer having a range of $-130°$ to $+40°$C.

Tissues to be dried were less than 3 mm in greatest dimension and were rapidly frozen in isopentane cooled by liquid nitrogen. One fragment was frozen with the thermocouple in place. The specimens were rapidly transferred to the apparatus. The cold finger was filled with liquid nitrogen and the main trap surrounded by a Dewar flask containing liquid nitrogen. The tissue holder was placed in a Dewar flask containing alcohol/dry ice mixtures at various temperatures or a diethyl oxalate/dry ice mixture. Within 15 minutes after starting the pump, the pressure

[1] This work supported by research grant #G3326 from the National Science Foundation.
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had reached the range of the ionization gauge and the following routine was followed: (1) A pressure reading was taken at the end of the first hour while liquid nitrogen remained within the cold finger. (2) The liquid nitrogen was then rapidly removed from the finger by suction and the trap filled with acetone at room temperature. The highest pressure reached immediately after removal of the liquid nitrogen was noted. (3) Finally, an equilibrium pressure was recorded 10 minutes after the liquid

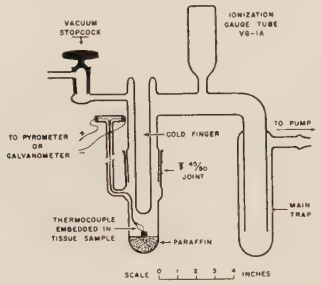

Fig. 1.

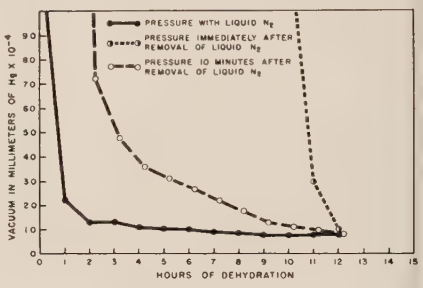
PRESSURE WITH LIQUID N2
PRESSURE IMMEDIATELY AFTER REMOVAL OF LIQUID N2
PRESSURE 10 MINUTES AFTER REMOVAL OF LIQUID N2
VACUUM IN MILLIMETERS OF Hg X 10⁻⁴
HOURS OF DEHYDRATION

Fig. 2.

Fig. 1.—Freeze-drying apparatus modified after Glick and Malmström.

Fig. 2.—Pressure recordings during dehydration of 3 pieces of liver at −30°C. The sharply defined drying endpoint is illustrated.

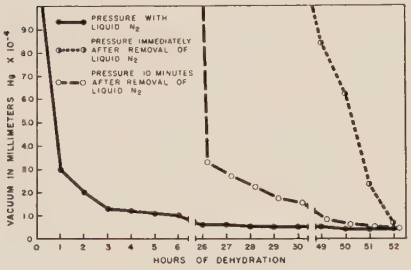
PRESSURE WITH LIQUID N2
PRESSURE IMMEDIATELY AFTER REMOVAL OF LIQUID N2
PRESSURE 10 MINUTES AFTER REMOVAL OF LIQUID N2
VACUUM IN MILLIMETERS Hg X 10⁻⁴
HOURS OF DEHYDRATION

Fig. 3.—Pressure recordings during vacuum dehydration of 20 pieces of myocardium at −30°C. Note that the drying endpoint is sharply defined at 52 hours.

nitrogen was first removed. The cold finger was again filled. Tissue temperatures at the beginning and end of each cycle were recorded. The above operations were repeated each hour until all three pressures were equal. Dehydration was considered complete only when the pressure spike, resulting from the rapid removal of the liquid nitrogen and warming of the cold finger, was no longer elicited. Once drying was complete, the paraffin was melted and the tissue embedded *in vacuo*.

Discussion.—The procedure outlined above provides a satisfactory tissue dehydration endpoint. This was substantiated by the absence of bubbling from the tissue upon embedding *in vacuo* and by examination of sections. Two actual records are presented in Figs. 2 and 3. The former illustrates the drying curve of three pieces of liver weighing 0.19 g. Fig. 3 represents the plotted pressures of 20 samples of myocardium with an original wet weight of 0.96 g. Embedding of tissues prior to the graphic endpoint resulted in bubbling of the samples with the subsequent observation of

areas of incomplete drying within microscopic sections. As can be seen from Figs. 2 and 3, it is only the pressure peak which occurs as a result of the sudden warming of the cold finger that has a rapid enough rate of change to be used as a well defined endpoint. The extreme sensitivity of this method is due to the fact that it is dependent upon the accumulation of water over a period of time and its sudden release. Thus, although it is not capable of measuring absolute rates of sublimation as is the method used by Naidoo and Pratt [13], it has the advantage of much simpler design without the necessity of a valve between the tissue and the trap.

For practical purposes, it was noted that it was necessary only to release the sublimed water from the cold finger trap at irregular intervals to provide an indication of how close the tissues were to complete dryness. It was observed, however, that the total drying time was shorter by several hours when the accumulated water was released at hourly intervals. This was probably a result of the increased efficiency of the trapping surface. The periodic warming of the cold finger did not raise the temperature of the piece of tissue containing the thermocouple in an unpredictable manner. It approached but did not reach the level of the surrounding bath, falling again once liquid nitrogen was restored to the trap. During the process of drying, the temperature of the tissue followed the same general curve described by Jansen [10].

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THE EFFECTS OF VARIOUS METABOLITES AND ANTI-METABOLITES ON THE REGENERATION OF FRAGMENTS OF *ACETABULARIA MEDITERRANEA*

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Received May 3, 1958

The effects of various purine and pyrimidine bases (adenine, uracil, hypoxanthine, thymine, cytosine, orotic acid, alone or in mixture), nucleosides (adenosine, guanosine, uridine, thymidine, cytidine, alone or in mixture), amino acids (methionine, phenylalanine, cysteine, arginine) have been compared with those of chemical analogues of these compound (2–6 diaminopurine, thiouracil, 5-aminouracil, azaguanine, benzimidazole, fluorophenylalanine, ethionine, kinetin) on the regeneration of nucleate and anucleate fragments of *Acetabularia mediterranea*. The action of chloramphenicol (which inhibits amino acid incorporation in isolated nuclei hardly at all) [1], and of the –SH containing, strongly reducing compound β-mercaptoethanol [4] has also been studied. All the substances tested were used at a 10^{-3} M concentration (or at saturation, if their solubility was less than that), except for chloramphenicol (2–10 μg/ml) and mercaptoethanol ($M/300$).

The main results obtained can be summarized as follows:

(1) A paradoxical effect was found regularly in that the chemical analogues were *less* inhibitory and toxic than the normal nucleic acid bases or amino acids. Among the former, adenine and hypoxanthine, and among the latter, arginine, were especially toxic. In general, nucleosides were less inhibitory than free purines and pyrimidines. Obviously, nucleic acid and protein metabolism must be well balanced in order to allow growth and regeneration. As a rule, anucleate fragments were found to be more sensitive to these toxic and inhibitory agents than the nucleate ones.

(2) Chloramphenicol, at the concentration tested, had no effect at all on growth and regeneration whether the fragments were nucleate or anucleate.

(3) Kinetin, which stimulates cell division in plant cells, had a slight inhibitory effect on growth and regeneration, comparable to that exerted by the purine and pyrimidine analogues.

(4) Thymine and thymidine deserve special mention, because they are normal precursors of DNA, a substance that is apparently present in *Acetabularia* in such small amount that it cannot be detected. Thymine proved to be more inhibitory to growth and regeneration than thymidine. Autoradiography and counting experiments, using C^{14}-labelled thymine and tritium-labelled thymidine showed a rapid uptake in both nucleate and anucleate halves. Most of the radioactivity was found in the RNA fraction, especially in uridylic and cytidylic acids. These results cast some doubt on the absolute specificity of thymine and thymidine as precursors of DNA.

(5) In 3 experiments out of 4, ethionine had an initial stimulating effect on cap formation, only in anucleate halves. Later, however, a large proportion of these

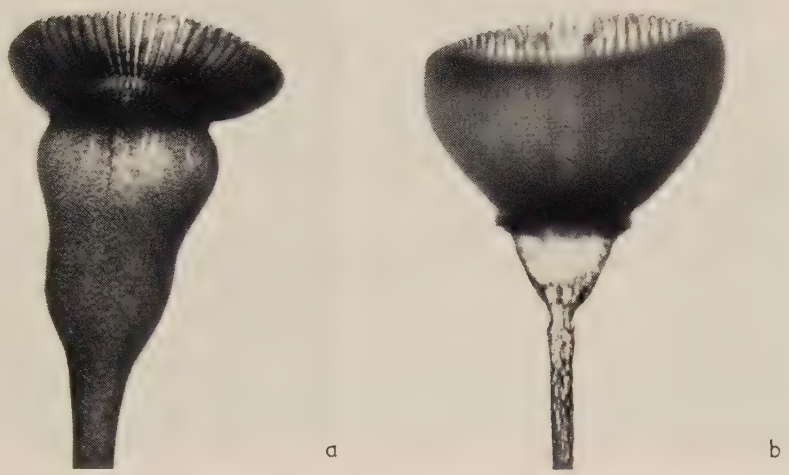

Fig. 1.—Abnormal caps formed by anucleate halves treated with ethionine (10^{-3} *M*). There is no cap formation when nucleate halves are treated in the same way.

caps became highly abnormal (Fig. 1). Ethionine strongly inhibits regeneration in nucleate halves: it was the only substance found to be more inhibitory to nucleate than anucleate halves.

(6) Mercaptoethanol, but not cysteine or glutathione, exerted a very powerful inhibitory effect on cap formation, in both nucleate and anucleate pieces. This substance has little toxicity at the concentration used, the effects being similar to those obtained when the amount of light allowed the alga is reduced [2].

Taken together, the observations on the effects of ethionine and mercaptoethanol suggest that *sulfur metabolism* might play an important role in the synthesis of the *specific cap-forming morphogenetic substances* [3]. However, the possibility that mercaptoethanol acts in an indirect manner (by reducing intracellular oxygen tension, for instance) rather than by a specific reduction of protein –SS– bonds, cannot for the present be excluded.

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